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Javier A. Menendez & Ruth Lupu

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REVIEW

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Fatty acid synthase (FASN) as a therapeutic target in breast cancer

Javier A. Menendez[a,b] and Ruth Lupu[c,d]

aProCURE (Program Against Cancer Therapeutic Resistance), Metabolism & Cancer Group, Catalan Institute of Oncology, Girona, Spain; bGirona Biomedical Research Institute (IDIBGI), Parc Hospitalari Martí i Julià, Girona, Spain; cDepartment of Medicine and Experimental Pathology, Mayo Clinic, Rochester, MN, USA; dMayo Clinic Cancer Center, Rochester, MN, USA

ABSTRACT

Introduction: Ten years ago, we put forward the metabolo-oncogenic nature of fatty acid synthase (FASN) in breast cancer. Since the conception of this hypothesis, which provided a model to explain how FASN is intertwined with various signaling networks to cell-autonomously regulate breast cancer initiation and progression, FASN has received considerable attention as a therapeutic target. However, despite the ever-growing evidence demonstrating the involvement of FASN as part of the cancer-associated metabolic reprogramming, translation of the basic science-discovery aspects of FASN blockade to the clinical arena remains a challenge.

Areas covered: Ten years later, we herein review the preclinical lessons learned from the pharmaceutical liabilities of the first generation of FASN inhibitors. We provide an updated view of the current development and clinical testing of next generation FASN-targeted drugs. We also discuss new clinico-molecular approaches that should help us to convert roadblocks into roadways that will propel forward our therapeutic understanding of FASN.

Expert opinion: With the recent demonstration of target engagement and early signs of clinical activity with the first orally available, selective, potent and reversible FASN inhibitor, we can expect Big pharma to revitalize their interest in lipogenic enzymes as well-credentialed targets for oncology drug development in breast cancer.

1. Introduction

Three decades after Otto Warburg ignited interest in how metabolism in tumor cells differs from that in normal cells in terms of exacerbated glycolytic carbon flux [1], Medes et al. (1953) were the first to show that tumors could also convert glucose or acetate into *de novo* synthesized lipids at a relatively low, but similar rate to that found in major lipogenic tissues such as liver [2]. It was not until the mid-1980s, however, that the prevailing notion that rapidly growing tumors obtain all their lipids performed by lipogenic host tissues in a non-cell autonomous manner evolved into a new paradigm where cancer cells, in a cell autonomous manner, seemed to generate at least a portion of their fatty acid (FA) content through *de novo* biogenesis [3]. A landmark study by Kuhajda et al. (1994) established that OA-519, a prognostic molecule in tumors from patients with breast cancer with markedly worsened prognosis, was actually a key, rate-determining enzyme for *de novo* FA biogenesis, namely fatty acid synthase (FASN) [4].

Upregulation of FASN accompanies the natural history of most human cancers, including breast carcinomas. FASN activation is an early and near universal hallmark of most human carcinomas and their precursor lesions [5–7], and is increased in a stage-dependent manner that is associated with worsened patient survival [8–17]. A FASN status-prognosis relationship strongly suggests that FASN-catalyzed endogenous lipogenesis should confer growth and survival advantages to cancer cells. Similar to other molecular facets of deregulated cellular metabolism in tumor tissue, the lipogenic role of FASN in tumors has been perceived as an indirect, secondary phenomenon triggered by upstream signaling pathways (e.g. PI3K-AKT-mTOR and MAPK) commonly activated by different cancer-driven genetic lesions (Figure 1) [18–21]. The notion that FASN overexpression/hyperactivation is only required to support oncogene-directed anabolic proliferation and survival [22–26] has been challenged by the recognition that FASN signaling can regulate not only cell proliferation, cell survival, cell adhesion, extracellular matrix (ECM) organization, migration, and invasion, but also the expression and activity of oncogenic proteins closely related to malignant transformation (Figure 1) [27]. The ability of FASN-catalyzed endogenous lipogenesis to interact with and regulate multiple cancer-controlling networks [27–30] along with the discovery that FASN overexpression suffices to drive malignant-like phenotypes in epithelial cells [31,32] led to the suggestion in 2007 that FASN can operate as an oncogene-like factor [33–35].

Since the conception of the metabolo-oncogenic nature of FASN 10 years ago [35], which provided a molecular framework to explain how FASN signaling might cell-autonomously regulate cancer initiation and progression, FASN has received considerable attention as a therapeutic target. While several

CONTACT Ruth Lupu ✉ lupu.ruth@mayo.edu 🖃 Mayo Clinic Cancer Center, 200 First Street SW, Rochester, MN 55905, USA; Javier A. Menendez ✉ jmenendez@idibgi.org
🖃 Girona Biomedical Research Institute (IDIBGI), Parc Hospitalari Martí i Julià, 17190 Girona, Spain

Article highlights

- The lipogenic enzyme FASN is part of the metabolic reprogramming cancer hallmark.
- Most of the first generation FASN inhibitors described in the literature should be viewed as tool compounds rather than clinically valuable oncology drugs.
- The apparent discrepancy between *bench* findings and the awaited *bedside* effects has remained an elusive challenge until recently.
- We are celebrating the fact that next generation FASN-targeted drugs with optimized pharmacological properties and *in vivo* tolerability has just entered the clinic.
- We anticipate that additional FASN inhibitors will be integrated into an expanding pipeline of targeted drugs based on an ever-growing understanding of the FASN biology-breast cancer association.
- We can expect big pharma to revitalize their interest in lipogenic enzymes as well-credentialed targets for oncology drug development in breast cancer.

This box summarizes key points contained in the article.

different FASN inhibitors have been developed and comprehensively characterized in molecular and cell-based preclinical studies, most of the inhibitors described in the literature should be viewed as tool compounds rather than clinically valuable oncology drugs [36–39] (Figure 2). Despite ever-growing evidence demonstrating the involvement of FASN signaling in metabolic reprogramming in cancer, translating the basic science-discovery aspects of FASN blockade to the clinical arena has remained an elusive challenge until recently. The pharmaceutical liabilities of first-generation FASN-targeting compounds are beginning to be circumvented with the discovery of next-generation FASN inhibitors with optimized pharmacological properties and *in vivo* tolerability [40,41]. One

of them, the first oral, selective, and potent reversible FASN inhibitor TVB-2640, has recently entered clinical trials (Figure 2).

Here, we review the clinico-molecular lessons learned from the pharmaceutical liabilities of first-generation FASN inhibitors in the last decade, and provide an update on the current development and testing of next-generation FASN-targeted drugs. We also discuss new clinico-molecular approaches that should help to turn potential roadblocks into roadways to propel forward our therapeutic understanding of FASN in breast cancer.

2. First-generation FASN inhibitors: more pitfalls than promises

A quick search for FASN and breast cancer on PubMed.gov today yields a listing of almost 300 publications, 180 of them since 2007. The impression that arises from the literature is that substantial research efforts have been focused in developing strategies to target FASN in breast cancer. Indeed, a variety of FASN inhibitors aimed to exploit the lipogenic dependency of breast cancer have been developed in the last decade (Figure 3).

2.1. Cerulenin and C75

The natural product cerulenin, an antibiotic originally isolated from *Cephalosporium caerulens*, was one of the first compounds found to inhibit FASN activity, by forming an adduct with an active-site cysteine in the FASN β-ketoacyl-synthase domain [45]. Cerulenin inhibits proliferation and induces programmed cell death in breast cancer cells *in vitro*, and delays
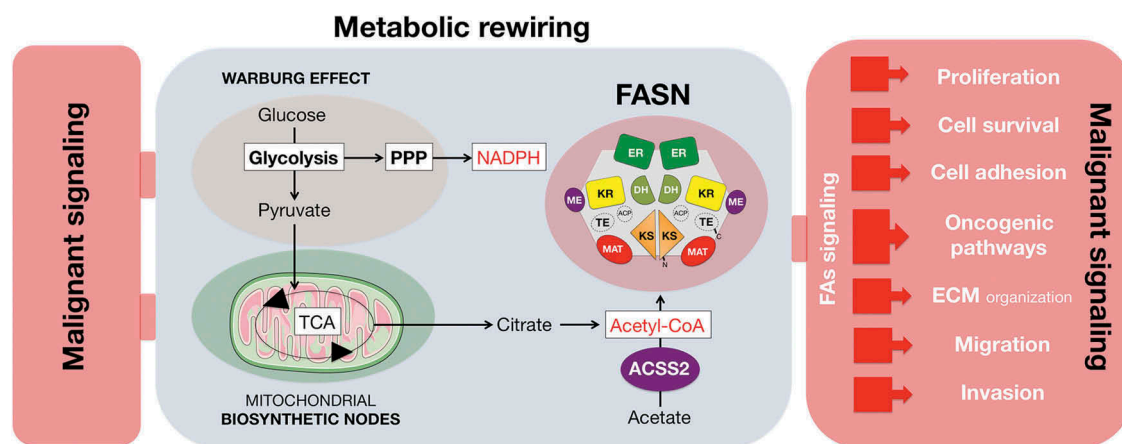

Figure 1. The metabolo-oncogenic nature of FASN in breast cancer: Breast cancer cells might exhibit increasing autonomy in maintaining an aberrant metabolic phenotype including hyperactivation of FASN signaling because proto-oncogenes and tumor-suppressors originated through evolution as early components of metabolic regulation networks. Breast cancer-associated FASN might therefore be viewed as an evolutionary conserved consequence of the metabolic rewiring that is upstream programed by oncogenic gain-of-function events and the loss of tumor-suppressors. A new developing paradigm begins to support the notion that activation of FASN signaling coupled to well-known cancer-related metabolic alterations such as the activation of the glycolytic Warburg effect and of biosynthetic nodes within mitochondria can be better understood in terms of upstream metabolic facilitators that operate as roadways for the molecular logic that ultimately orchestrate the signaling paths generating, maintaining, and facilitating the evolution of the malignant phenotype. Palmitate, the end product of FASN, can be modified into a variety of lipids (e.g., phospholipids, triglycerides, cholesterol esters) and incorporated into fatty-acylated proteins, thereby providing essential components of cell membranes, significant substrates for energy metabolism, and signaling factors in post-translational modifications, all of them playing important roles in multiple stages of breast cancer progression. FA synthesis by FASN has been also shown to protect cells from apoptotic cell death while regulating metastasis-related ECM organization, migration, and invasion. FASN signaling also cross-talks with cancer-controlling networks to cell-autonomously regulate the expression and activity of oncogenic proteins closely related to breast cancer initiation and progression (e.g., HER2, ER). (PPP: Pentose phosphate pathway; TCA: Tricarboxylic acid cycle; ACSS2: acetyl-CoA synthetase 2).
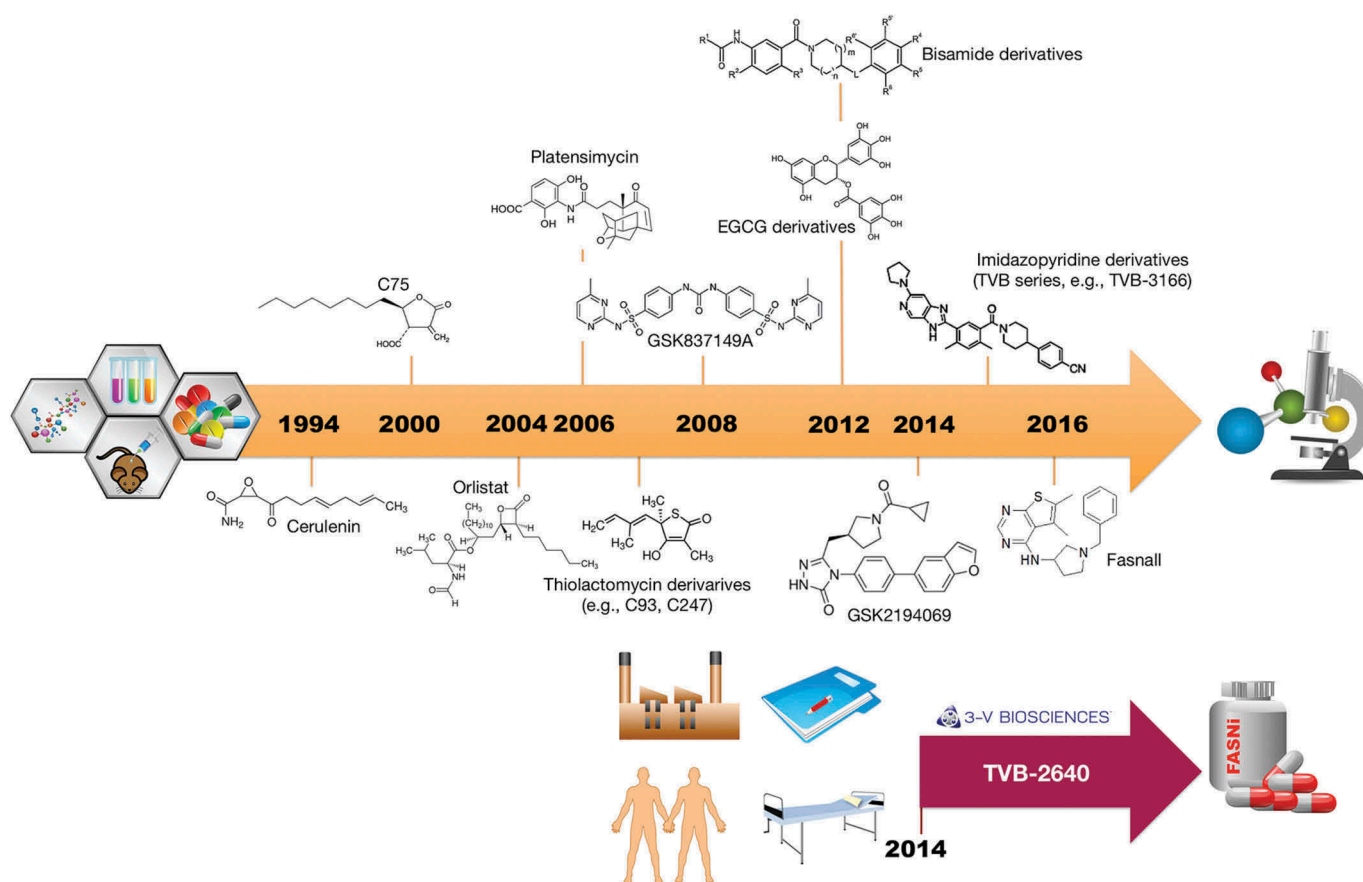
Figure 2. Lost in FASN translation: from pre-clinical testing to first-in-human clinical trials. The metabolo-oncogenic nature of FASN should form the basis to pursue unique therapeutic approaches (i.e., FASNinhibs – see Figure 3-) that target the addiction of breast cancer cells to the FASN-centered signaling infrastructure. Although substantial pre-clinical efforts have been made to develop strategies to target FASN for breast cancer treatment (upper timeline), only one targeted compound aimed at blocking FASN (TVB-2640 developed by 3-V Biosciences) has so far entered clinical trials (bottom timeline).
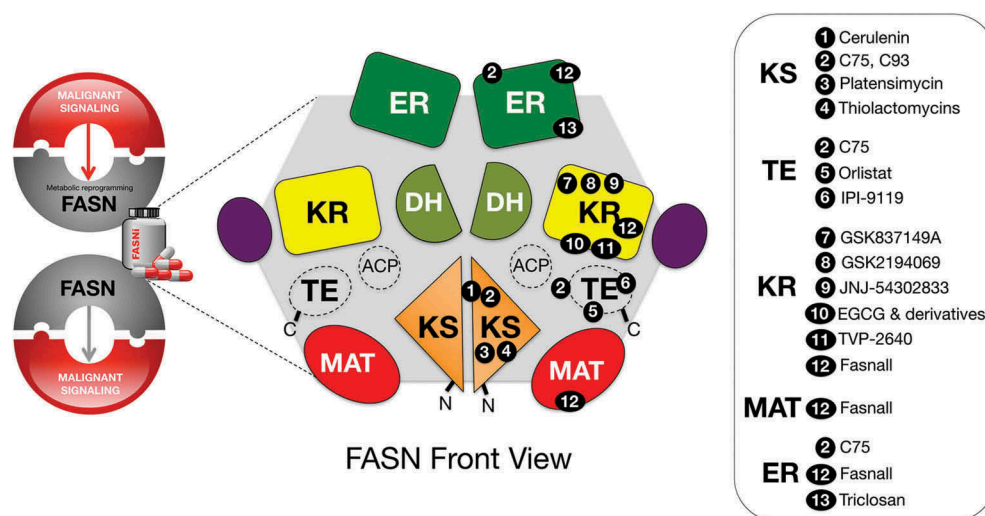

Figure 3. FASNinhibs and FASN domains: a structural overview. Mammalian FASN consists of two identical multifunctional polypeptides, each including seven catalytic domains: β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and thiosterase (TE). Figure shows a cartoon representation (front view) of the X-shaped structure of FASN [42–44] highlighting the interactions of FASNinhibs with a full set of druggable active sites present in each of the two 'arms' on both sides of the molecule, i.e., a 'selection/condensing' arm (KS and MAT domains) for addition of new building blocks into the nascent FA chain, and a 'modifying arm' (DH, ER, and KR domains) for chemical processing of FA chain elongation intermediates.

disease progression in cancer xenografts in a FASN-dependent manner [4,46–48]. The semi-synthetic compound C75, which was developed to circumvent the chemical instability of cerulenin's epoxy group [49], has multiple sites of interaction with FASN, and operates as a weak, irreversible FASN inhibitor through its interaction not only with the β-ketoacyl-synthase domain but also with the enoyl reductase and the thiosterase domains [50]. C75 can induce apoptosis and anti-tumorigenic

activity in multiple cancer cell lines and xenograft models [51–56], and prevent mammary cancer development in HER2 (neu-N) transgenic mice [57]. Unfortunately, a major setback in the use of cerulenin or C75 derivatives to target breast cancer-associated FASN was the finding that both compounds severely reduce food intake and induce body weight loss in mice [58–60].

2.2. Thiolactomycin derivatives

To overcome the lack of potency and off-target activities of cerulenin-based inhibitors, such as activation of β-oxidation and excessive energy expenditure, naturally occurring thiolactomycins have been used to develop a new class of synthetic FASN inhibitors with no weight-loss or anorexigenic side effects [61]. Representative of this class is C93 (or FAS93), which has significant antitumor activity against non-small cell lung and ovarian cancer xenografts as well as robust chemopreventive effects in chemically induced lung tumors [62–64]; however, little is known about its activity in breast cancer models. The C93-related compound, C247, has significant efficacy in a transgenic model of breast cancer [57,61,65]. FAS31, a new FASN inhibitor with oral bioavailability, exerts significant antitumor activity in ovarian cancer xenograft models with no observable toxicity to normal rat or mouse tissues and no significant effects on bodyweight [66]. While C93 has been shown to inhibit the β-ketoacyl-reductase activity of FASN, nothing is known about the actual structures and ultimate mechanisms of action of C247 and FAS31.

2.3. Small-molecule inhibitors from medicinal chemistry programs and high-throughput screening

Big pharmaceutical companies including Merck, AstraZeneca, and GlaxoSmithKline have shown interest in discovering new small-molecule FASN inhibitors through medicinal chemistry programs and high-throughput screening. Such approaches have led to the development of more potent FASN inhibitors with activities in the low nanomolar range, including a series of 3-aryl-4-hydroxyquinolin-2(1H)-one derivatives, bisamide derivatives, the dibenzenesulfonamide urea GSK837149A, and the bacterial FabF/B inhibitor platensimycin [67–71]. Though GSK837149A can irreversibly inhibit the β-ketoacyl reductase domain of FASN in biochemical studies, experiments in cancer cell lines were not possible because of its extremely poor cell permeability [68]. Unfortunately, nothing is known about the mechanisms of action and cellular in vivo activity of Merck and AstraZeneca FASN inhibitory scaffolds, or about the potential activity of platensimycin against mammalian FASN in cancer cell lines.

2.4. Orlistat

Orlistat (ORL), a US FDA-approved pancreatic lipase inhibitor originally developed as an anti-obesity drug, is a potent irreversible inhibitor of FASN via its ability to form a covalent adduct with the active serine of FASN thiosterase domain [72,73]. While ORL has shown some tumor growth inhibition activity in xenograft models of prostate cancer and in mouse

melanoma models [74–76], its antitumoral actions on breast cancer is limited to one study showing reduced proliferation and exacerbated apoptosis in HER2 oncogene-overexpressing breast cancer cells [77].

Given the important pharmacological limitations of ORL, including poor oral bioavailability and metabolic stability, and lack of selectivity, several attempts have been made to develop ORL derivatives with improved solubility and increased potency [78–82]. To circumvent the hydrophobicity and low systemic uptake of orally administered ORL, a new nanoparticle (NP) formulation of ORL using amphiphilic bio-conjugates derived from hyaluronic acid has recently been developed [83]. The so-called Nano-ORL has been shown to retain similar levels of FASN inhibition while having significantly improved cytotoxicity in triple-negative breast cancer (TNBC) models [83]. Similarly, the use of hydrophilic poly(ethylene glycol)-conjugated poly(lactic-co-glycolic acid) nanoparticles (PLGA-PEG-NPs) as delivery system has improved the cytotoxic activity of ORL against TNBC cells by improving bioavailability [84]. Moreover, folate receptor-targeted micellar NPs carrying ORL have been shown to significantly improve the water-solubility, delivery, and bioavailability of ORL to TNBC cells growing in culture and in tumor xenografts [85]. These findings, collectively, appear to indicate that NP-based packaging might accelerate the development of new ORL formulations for TNBC, a subtype of breast cancer characterized by aggressive behavior, distinct patterns of metastasis, and lack of targeted therapies [86].

3. Next-generation FASN inhibitors: avoiding pitfalls

While ever-growing basic research has provided clear support for FASN as a breast cancer target, there is apparent discrepancy between bench findings and the awaited bedside effects in clinical trials. Indeed, none of the aforementioned FASN inhibitors has been tested in patients with cancer because of limitations imparted by their pharmacological properties or side-effect profiles. However, despite the challenges of selectively inhibiting FASN-driven lipid metabolism without major systemic effects, we are celebrating the fact that a new generation of FASN inhibitors has just entered the clinic.

3.1. TVB-3166

TVB-3166 is a member of a new generation of highly potent, reversible, imidazopyridine-based FASN inhibitors discovered and developed by 3-V Biosciences (http://www.3vbio.com), which inhibit de novo palmitate synthesis in vitro and in vivo [40,87–89]. Using in vitro and in vivo human carcinoma models including breast cancer, Ventura et al. (2015) revealed that FASN inhibition by TVN-3166 has multifaceted, unreported mechanisms [40] that apparently differentiate it from the archetypal FASN inhibitors cerulenin and C75 [89]. TVN-3166-induced inhibition of de novo FA biogenesis was found to disrupt lipid raft architecture and promote mislocalization of membrane-associated molecules and signaling pathways including Ras, AKT-mTOR, and Wnt-β-catenin that ultimately caused tumor cell apoptosis. Because signal transduction via such molecules is tightly linked to glucose and glutamine

tumor cell metabolism and lipid biosynthesis, it would appear that the sole inhibition of *de novo* palmitate synthesis by TVN-3166 is sufficient to concurrently block metabolic and signal transduction pathways vital to cell growth, proliferation, and survival in a tumor-specific manner [40]. Importantly, the ability of TVN-3166 to globally reprogram gene expression included the inhibition of core oncogenic effectors such as c-Myc. All these phenomena occurred without activation of FA oxidation or other undesirable off-target effects. Moreover, a significant inhibition of tumor growth in xenograft models occurred with once-daily dosing of TVB-3166, which was capable of inhibiting FASN activity for approximately 10–12 h each day, revealing that continuous target engagement is not required for achieving *in vivo* tumor growth inhibition.

3.2. TVB-2640: the only clinically available FASN inhibitor

The definitive demonstration that targeted FASN inhibition can achieve the expected anticancer effects in a well-credentialed target in oncology is just emerging with a first-in-class clinical trial using the only clinically available FASN inhibitor, TVB-2640 (Figure 2). A phase I, first-in-human study of escalating doses of oral TVB-2640 in patients with solid tumors (ClinicalTrials.gov: NCT 02223247) is currently enrolling patients with advanced solid tumor malignancies. The objectives of this trial are to determine the maximum tolerated dose, the recommended phase 2 dose (RP2D in monotherapy and in combination with chemotherapy), and to explore the safety profile of TVB-2640 as monotherapy and in combination with weekly paclitaxel (PXT). TVB-2640 is being administered as a once-daily oral agent: 21 days in monotherapy or 28 days with PXT in continuous cycles. TVB-2640 has a favorable tolerability profile with no significant gastrointestinal, hematological or serum chemistry adverse events, and no abnormal QTc prolongation. In a recent update of the trial [90], patients were treated with doses of TVB-2640 ranging from 60 to 240 mg/m^2, as well as with flat doses of 200 and 250 mg/m^2. Dose-limiting toxicities occurred at levels of 120 and 240 mg/m^2 and included two grade 3 ocular toxicities (iritis and corneal edema) and two skin toxicities (both were hand-foot syndrome), which were considered on-target effects and were reversible with drug discontinuation. Though not observed in monotherapy, symptomatic pneumonitis was observed in breast cancer patients treated with TVB-2640 in combination with PXT.

The pharmacokinetic (PK) profile of TVB-2640 is favorable, with plasma levels increasing with dose, steady-state levels reached by day 8, and a half-life of approximately 15–16 h that remains unaffected by concurrent PXT. Remarkably, FASN inhibition modeling at a TVB-2640 exposure of 60 mg/m^2 and above demonstrated that FASN target modulation exceeded the minimum threshold for preclinical efficacy in all but one patient. The RP2D has been defined as 100 mg/m^2. Preclinical data and early efficacy data from the dose-escalation trial has shown broad monotherapy activity in multiple solid tumors, including multiple cases of stable disease (SD). Breast cancer preliminary antitumor data has shown that TV-2640 combined with weekly PXT resulted in multiple RECIST partial responses and prolonged SD in 93% of patients treated [91]. Further exploration in dose expansion cohorts for the combination therapy will be pursued in breast, ovarian, and non-small cell lung cancer.

3.3. Fasnall

All the aforementioned FASN inhibitors, including the recently described GSK2194069, a potent and specific inhibitor of the β-ketoacyl reductase activity of FASN [92], share a common molecular behavior that favors competition with substrate intermediates over cofactor binding. To circumvent the current limitations of the substrate domain-based discovery of FASN inhibitors, new scaffolds specifically targeting the largely unexplored cofactor domain sites have been recently developed [93]. Because three of the FASN enzymatic activities (ketoacyl reductase, enoyl reductase, and malonyl/acetyltransferase) use purine-containing cofactors in the form of NADPH, acetyl-CoA, and malonyl-CoA, Alwarawrah and colleagues [93] recently took advantage of an innovative fluorescence-linked enzyme chemoproteomic strategy [94] to specifically identify new FASN inhibitors targeting the nucleotide-binding pockets. They identified Fasnall, a thiophenopyrimidine-based FASN inhibitor with potent and broad antitumor activity against various breast cancer cell lines. Global lipidomic studies revealed some mechanistic peculiarities of Fasnall, including a sharp increase in the intracellular levels of ceramides, diacylglycerols, and unsaturated FAs, with the increase in ceramides contributing, at least in part, to Fasnall-induced apoptotic cell death. Intriguingly, Fasnall treatment appeared to recover the lipid storage function of endogenous FA biogenesis normally occurring in lipogenic cells. Correspondingly, Fasnall-treated breast cancer cells exhibited a significantly increased uptake of exogenous palmitate that was directed more into neutral lipid formation rather than into phospholipid signaling molecules [93].

The unique ability of Fasnall to inhibit the FASN-facilitated production of phospholipids with saturated acyl chains while promoting the uptake of exogenous unsaturated FAs may drastically affect lipid raft structure and functioning, suggesting that lipid rafts might constitute a common target for structurally and mechanistically unrelated FASN inhibitors such as cerulenin/C75, TVB-3166, and Fasnall [23,94,95]. Nevertheless, Fasnall has a significant *in vivo* antitumor activity in both the clinically relevant HER2+ MMTV-Neu and the TNBC C3Tag breast cancer mouse models, which is synergistically augmented in terms of reduced tumor volumes and affected survival when combined with the platinum-based chemotherapeutic agent carboplatin. That Fasnall was well tolerated without inducing any change in feeding behavior or weight loss in mice, together with the adaptability of the synthetic route of Fasnall for the preparation of new analogs, strongly suggest that the Fasnall scaffold can be developed further to optimize its pharmacological properties *in vivo* [93].

4. Natural and indirect sources of FASN inhibition

4.1. Plant-derived polyphenols

A growing list of natural plant-derived polyphenols have inhibitory action against FASN activity and expression, including epigallocatechin-3-gallate (EGCG), the flavonoids luteolin, taxifolin, kaempferol, quercetin, and apigenin, and extra virgin olive oil secoiridoids [96–101]. Unlike luteolin, which exhibits the greatest effect on lipogenesis of the plant-derived polyphenols via direct and indirect inhibitory actions on FASN [100,101], the natural component of green tea, EGCG, one of the best characterized polyphenols with FASN inhibitory activity that does not promote anorexia and weight loss [102], might solely produce blockade of the β-ketoacyl reductase domain of FASN in a high micromolar range [96,99], thus greatly limiting its further development as a FASN inhibitor. A novel family of more potent EGCG analogs has been developed [103–106] and preclinical approaches have shown that such series of polyphenolic compounds might exert antitumor activity against HER2+ models of breast cancer without exhibiting cross-activation of β-oxidation or inducing weight loss [107,108].

4.2. Metformin

Accumulating epidemiological, preclinical, and clinical evidence demonstrates that the biguanide metformin (1,1-dimethylbiguanide hydrochloride), the first-line drug treatment for type 2 diabetes, is a promising candidate for oncology therapeutics [109–111]. While the underlying mechanisms of action of metformin against tumor cells remain elusive [112–115], its multifaceted nature has been shown to involve the inhibition of FASN in TNBC [116]. The ability of metformin to operate as a low-energy-mimicker capable of activating AMP-activated protein kinase (AMPK) by increasing the AMP: ATP ratio might lead to a reduction in the expression of the master lipogenic transcriptional regulator, SREBP-1c, therefore deactivating the lipogenic phenotype by coordinately suppressing the expression of acetyl-CoA carboxylase, FASN and other enzymes that regulate endogenous lipid biogenesis [117,118]. However, metformin has also been shown to decrease the expression of several lipogenic enzymes and lipogenesis in an AMPK- and SREBP-1c-independent manner [119,120]. A recently proposed substrate limitation model of action, in which metformin restricts the production of mitochondrial-dependent biosynthetic intermediates, might explain its ability to deplete acetyl-CoA and malonyl-CoA precursors required for FASN-related *de novo* lipid biosynthesis [121–123].

5. Integrating new approaches for clinical development of FASN inhibitors

The first generation of FASN blockers (e.g. cerulenin, C75, ORL), although displaying potent cytotoxic effects *in vitro* and *in vivo*, suffered from limitations in selectivity as well as metabolic and pharmacological limitations that collectively hampered their use in clinical settings. The preclinical lessons learned from early FASN inhibitors might help to delineate new clinico-molecular approaches capable of sidestepping potential roadblocks in the therapeutic avenue for the treatment of breast cancer using next-generation FASN inhibitors.

5.1. Systemic toxicity and lack of in vivo efficacy of FASN inhibitors: mechanistic and physiological concerns

Toxicity and lack of *in vivo* efficacy of the majority of FASN inhibitors can be viewed as a consequence of their lipid-like nature, which allows them to act as competitors of FASN substrate intermediates [93]. The development of non-lipophilic Fasnall-like molecules selectively targeting FASN through its cofactor binding sites might significantly ameliorate the tolerability of newly developed FASN-targeted drugs. However, while it remains to be clarified whether TVB-2640 [90,91,124,125], the only FASN inhibitor that has entered the clinic, exclusively acts in a competitive manner with the substrate intermediate of the FASN β-ketoacyl reductase step, a new generation of FASN-targeted molecules including GSK2194069 and the spirocyclic imidazolinone JNJ-54,302,833 [92,126], both of which target the β-ketoacyl reductase domain, and the tetrazolone carboxamide analog IPI-9119, which irreversibly targets the thiosterase domain [127], have shown selectivity and reversible toxicities during target engagement *in vivo* without apparently involving competition with FASN co-factor binding sites.

Despite the fact that *de novo* FA biogenesis is restricted mainly to liver, adipose tissue, lactating breast, and cycling endometrium in humans [22,25,35,128,129], a commonly raised concern with FASN-targeting drugs relates to the physiological consequences and compensatory adaptive responses that might occur upon acute or chronic FASN inhibition. However, because *de novo* biosynthesis is not the main path through which adult tissues fulfill their lipid needs, it is reasonable to assume that most normal tissues should be protected from the toxic effects of targeting FASN-driven endogenous FA synthesis through lipids provided by the diet via bloodstream. Moreover, because fundamental differences in the ability of FASN to respond to normal FA regulatory actions in lipogenic tissues may account for the extremely high levels of FASN in subsets of breast carcinomas [130,131], dietary manipulations to alter the availability of the amount and type of serum-derived lipids in the tumor microenvironment may be a plausible strategy to restrain compensatory adaptive responses that would potentially mitigate the efficacy of FASN inhibitors *in vivo*. Thus, while metronomic treatment regimens might be suggested as a strategy to alleviate the role that highly lipogenic tissue might have in determining dose-limiting toxicities of next-generation FASN inhibitors [41], perhaps more attention should be paid to behavioral strategies in diet control (e.g. FA composition) to augment selective toxicity in FASN-inhibited cancer cells [132].

5.2. Combinatorial strategies with FASN-targeting drugs

Whilst efforts have largely focused on the identification of the best scenario for FASN-targeted monotherapy strategies,

accumulating evidence supports combinatorial strategies for treating breast cancer. FASN inhibitors have been shown to synergize with multiple chemotherapeutic agents including taxanes such as PXT and docetaxel [54,133], vinca alkaloids such as vinorelbine [134], antimetabolites such as 5-fluorouracil [135], platinum agents such as carboplatin [94], and anthracyclines such as doxorubicin [136]. Indeed, the TVB-2640 clinical trial NCT02223247 is enrolling patients treated in monotherapy or in combination with weekly PXT [137,138]. Furthermore, FASN inhibitors have been shown to restore the sensitivity of breast cancer cells with acquired resistance to chemotherapeutics such as doxorubicin [139,140] and molecularly targeted agents such as trastuzumab and lapatinib in HER2+ breast cancer models [141,142]. FASN overexpression-mediated palmitate overproduction has been proposed as a new mechanism of multidrug resistance involving changes in plasma membrane properties capable of protecting cells from endogenous and exogenous insults as well as promoting alterations in the intrinsic threshold of breast cancer cells for drug-induced apoptosis [139,140]. In this regard, it should be acknowledged that a major roadblock to the clinical-translational advance of FASN inhibitors is that the precise mechanism by which pharmacological interference with endogenous FA biogenesis facilitates apoptotic cell death remains unresolved. Recent observations from our laboratory have revealed a novel FASN-dependent mitochondrial priming that links de novo FA biosynthesis in FASN-overexpressing breast cancer cells to the intrinsic apoptotic threshold (manuscript in preparation). Mitochondria of FASN-inhibited breast cancer cells appear to exist in an apoptosis-prone state that might be exploited therapeutically through the use of FASN inhibitors in combination with apoptosis sensitizers (Figure 4).

5.3. Contextual lethality: a therapeutic opportunity for FASN inhibitors

We have learned from studies in mice with liver-specific inactivation of FASN that, whereas minimal phenotypic changes are detected on a regular diet, hyperglycemia and steatosis develop on a zero-fat diet or after prolonged fasting [143]. Because these results suggest that new fat generated from the FASN reactions regulates glucose, lipid, and cholesterol metabolism, future efforts should evaluate whether contextual inhibition of FASN might exacerbate the selective toxicity of FASN in subsets of breast cancer patients with high-fat/high-energy-related obesity. Although still unexplored in breast cancer, recent work from epidemiological studies of colon and prostate cancer suggests an interaction between obesity and the impact of FASN. The deleterious effects of FASN on survival seem to be more pronounced in obese patients [37,144–147], raising the possibility that women who are obese and whose breast tumors express high levels of FASN are more likely to obtain benefit from FASN inhibition. Given that metformin exhibits exacerbated FASN inhibitory properties in the context of the combined metabolic effects of available lipogenic acetyl-CoA and extracellular cholesterol [146], such contextual synthetic inhibition of FASN by metformin might enhance the efficacy of selective FASN inhibitors in obese breast cancer patients (Figure 4).

Contextual inhibition of FASN might also play a crucial role when antiangiogenic agents, rather than promoting vascular normalization instead promote chronic hypoxia [148]. Among the compensatory mechanisms that allow tumors to escape antiangiogenic-induced chronic hypoxia is the upregulation of FASN-related lipid anabolism [149]. In such a setting, FASN inhibitors might selectively trigger contextual lethality (Figure 4). The ability of antiangiogenics to induce vascular normalization or a hypoxic environment might be tractable
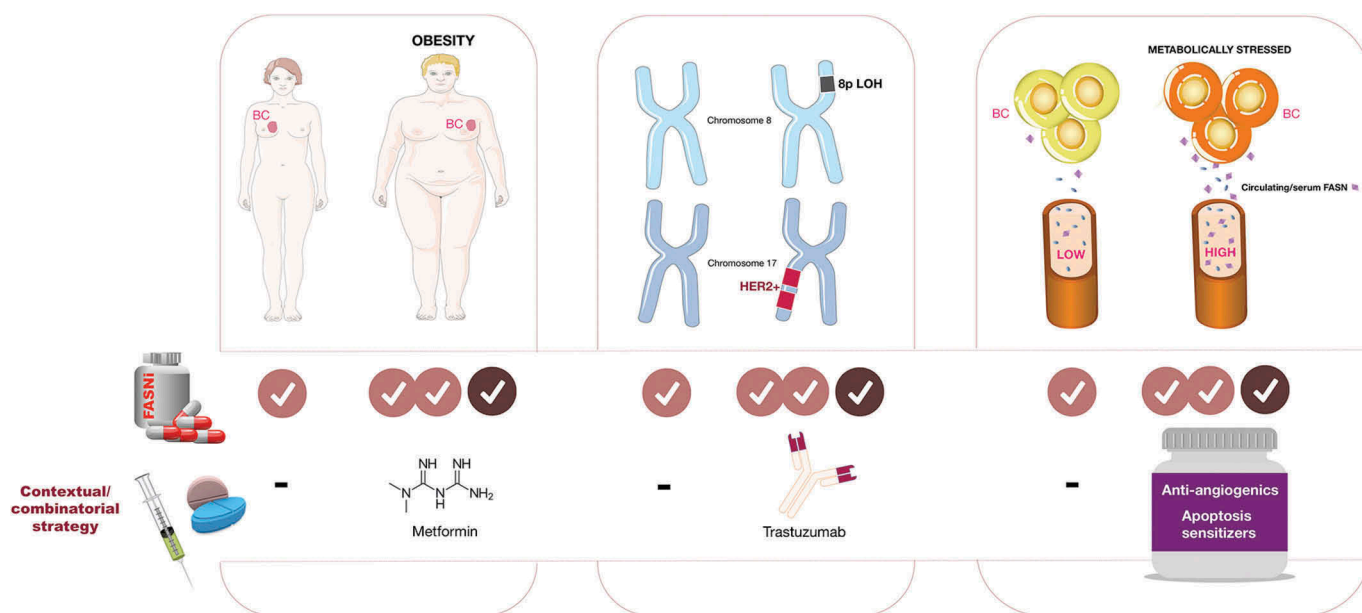

Figure 4. Selection and application of predictive markers for next-generation FASNinhibs: a predictive marker is a patient- or tumor-characteristic that better identifies results of treatment in particular subset of patients. Here, we propose molecular markers to identify breast cancer subsets that are likely to respond well to FASNinhibs (i.e., FASN-overexpressing breast carcinomas in obese woman, 8p LOH and HER2-gene amplified breast carcinomas, and metabolically-stressed breast carcinomas releasing high levels of circulating/serum FASN) and suggest a way to identify contextual/combinatorial strategies that might produce synergistic results when combined.

with ^{18}F-fluoromisonidazole-positron emission tomography (^{18}F-FMISO-PET) [150,151]; therefore, a noninvasive PET-tracer might be employed to detect baseline and antiangiogenic-induced hypoxic tumors that may respond to a FASN inhibitor when antiangiogenic treatment induces a hypoxic response, but not when the antiangiogenic agent induces normalizing effects.

6. Selecting breast cancer candidates to FASN inhibitors

Bridging the gap between a valuable oncology target such as FASN and the characteristics of breast cancer patients should guide the accurate convergence of laboratory-based experiments and clinical experience into well characterized, optimal subpopulations that would benefit from next-generation FASN inhibitors. Although the identification of pathophysiological mechanisms predictive of responsiveness to FASN inhibitors could greatly assist in prioritizing anti-FASN drug discovery resources, we should acknowledge that studies enabling a clear patient selection strategy are presently at the earliest stages of discovery. Recent evidence suggests that breast cancer-associated genomic alterations such as deletion of chromosome 8p significantly activates FA synthesis and confers tumor aggressiveness and chemotherapy resistance [152]; however, whether chromosome 8p loss of heterozygosity (LOH) might provide a genomic selection criteria to stratify breast cancer patients for treatment with next-generation FASN inhibitors remains to be tested (Figure 4).

Who might benefit from next-generation FASN inhibitors? Precise patient selection and identification of predictive biomarkers of response to FASN inhibitors is essential to ensure the measurement of true response rates without bias through inclusion of patients who fail to respond to FASN inhibitors simply because they are not suitable candidates to benefit from them. Although experimental data remain scarce, we are beginning to define some molecular features that might help to identify breast carcinomas that might be selected first for FASN inhibitor trials as they become available.

6.1. Expression status of the HER2 oncogene

Different breast cancer subtypes appear to varyingly employ cancer-associated metabolic traits including the FASN-driven lipogenic phenotype. Moreover, the differential usage of FASN-catalyzed *de novo* FA biogenesis seems to have diverse clinical implications for different breast cancer subtypes. Recent studies have shown a strong correlation between FASN overexpression and *HER2* oncogene amplification in breast cancer [153–155]. In our recent study, immunohistochemical staining for FASN in almost 200 cases of invasive ductal breast carcinoma confirmed a significant positive correlation with HER2 status; thus, a majority of clinically HER2+ tumors (85%) were scored as FASN overexpressors [55]. Moreover, the re-classification of HER2+ breast tumors based on FASN expression predicted a significantly inferior relapse-free survival and distant metastasis-free survival in the HER2+/FASN+ patient cohort. In a clinical setting where FASN-driven endogenous lipogenesis drives tumor cell proliferation, survival, and ultimately metastasis, selective FASN inhibition may be a valuable therapeutic strategy for the HER2+ breast cancer subtype. Indeed, HER2+/FASN+ breast cancer cells have repeatedly been shown to be exquisitely sensitive to first-generation FASN inhibitors such as cerulenin and C75 [23,26,27,31,53,55]. These findings, together with earlier discoveries showing that FASN blockade can restore sensitivity to trastuzumab and lapatinib in HER2+ breast cancer cells that acquired resistance to HER2-targeted therapies [141,142], strongly suggest that HER2 overexpression might be valued as a biomarker to delineate a subgroup of breast cancer patients that might benefit from therapeutic regimens containing FASN inhibitors (Figure 4).

6.2. Circulating levels of extracellular/serum FASN

Classically viewed as an intracellular protein, FASN can also be detected at increased levels in the extracellular milieu of cultured breast cancer cells as well as in the blood circulation of breast cancer patients [156–158]. Recent data are beginning to support the hypothesis that circulating extracellular FASN levels might increase in parallel with metabolic stress of the cells [159]. We found that extracellular levels of FASN were dependent on the metabolic state of the cells. Accordingly, AMPK-activating drugs mimicking metabolic stress promoted a dose- and time-dependent increase in extracellular FASN levels [159], suggesting that active secretion of FASN could be a physiologically relevant mechanism in the context of microenvironmental stresses such as nutritional deprivation or hypoxia. The presence of circulating FASN in patients with metabolic disorders such as over nutrition-induced insulin resistance, steatohepatitis, or chronic viral infections [160–163] supports the hypothesis that FASN secretion could be a regulated process to eliminate unnecessary FASN activity under conditions where lipogenesis is spared. In breast cancer tissues showing aberrant, constitutive upregulation of FASN expression due to genomic alterations, an increase of FASN levels in the blood might inform of an accelerated extrusion of the cytosolic enzyme under tumor microenvironmental conditions of depleted energy stores in response to nutrient starvation and/or biophysical stresses such as hypoxia. Indeed, immunohistochemical analyses of human breast tumors specimens indicate that FASN is strongly expressed in hypoxic regions [164]. Remarkably, in heavily pretreated breast cancer patients (with an average number of prior regimens including taxanes = 7) treated with TVB-2640 alone or in combination with PXT, 91% patients with high levels of serum FASN (≥10 ng/ml) benefited from prolonged SD whereas progressive disease was mostly observed in patients with low serum FASN (<10 ng/ml). It is tempting to suggest that if increased extracellular/circulating FASN levels are detected when tumors cannot meet increased energy demands (e.g. hypoxia or nutrient deprivation), it might constitute a bona fide surrogate marker of metabolically stressed breast carcinomas highly responsive to FASN inhibitors (Figure 4). Forthcoming prospective studies should confirm whether quantitative determination of FASN molecules in blood could become a rapid and accurate noninvasive test to identify and prioritize breast cancer patients for FASN inhibitor-based therapeutic intervention.

7. Measuring responses to FASN inhibitors

The sole source of preliminary biomarkers that might inform about tumor responses to FASN inhibitors are the PK/pharmaco-dynamic (PD) studies from the first-in-human, first-in-class, phase I clinical trial of TVB-2640 in patients with advanced solid tumor malignancies [90,91,124,137,138]. A key PD biomarker that has emerged in the development of TVB-2640 is serum malonyl carnitine, which appears to be reflective of the originally reported accumulation of malonyl-CoA after pharmacological FASN blockade [48,165]. A statistically significant >3-fold increase in serum malonyl carnitine was observed in a majority of patients by day 8 after initiation of TVB-2640 monotherapy for dose levels 120 and 240 mg/m^2. Indeed, increased serum malonyl carnitine was accompanied by decreased serum tripalmitin, a triglyceride derived from palmitic acid, in 90% of patients tested, thus confirming FASN engagement by TVB-2640.

We are lacking conclusive information regarding changes in serum FASN levels after treatment with TVB-2640. Circulating FASN has been suggested to be a bona fide biomarker of insulin sensitivity exclusively in the context of metabolic stress [162]. Normalization of circulating FASN was observed with an improvement in insulin sensitivity associated with increased bioenergetic efficiency (e.g. diet-induced weight loss and physical training-induce improvement of insulin sensitivity) or decreased intracellular FASN (i.e. surgery-induced weight loss), but not in response to fat accumulation and lipogenesis (i.e. pharmacological intervention with the AMPK/PPARγ activator rosiglitazone) [162]. The expected variability of serum FASN levels after treatment with FASN inhibitors should be carefully evaluated in terms of changes in FASN expression status and/or changes in key regulators of metabolic flexibility that might allow FASN-inhibited cells to switch from anabolic lipogenesis to catabolic β-oxidation of FAs or to rapidly switch from *de novo* biogenesis to lipid uptake. Accordingly, prospectively collected biopsies prior to treatment and post-dose after completing one cycle of TVB-2640 in the phase I NCT02223247 clinical trial have shown that changes in tumor FASN expression might occur after TVB-2640 therapy. Moreover, significantly increased reductions in phospho-active AKT were observed in TVB-2640-treated patients, including one patient with TNBC [90,91,124,137,138]. Because concurrent inhibition of FASN and of the PI3K/AKT signaling pathway promotes exacerbated levels of apoptotic cell death [166], the ability of FASN inhibitors to inactivate AKT in breast carcinoma tissues might inform about the incapacity of FASN inhibition-sensitive tumors to switch their addiction to a lipogenic phenotype upon FASN blockade.

8. FASN inhibitors to treat breast cancer: converting roadblocks into roadways

8.1. Tumor metabolic heterogeneity and innate resistance to FASN inhibitors

Intra-tumor heterogeneity, that is, the remarkable variety of cellular phenotypic traits in a given tumor, ranging from differentiation/proliferation states, migratory/invasive capacity, to size and therapeutic response [167–170], is probably the next big quest in cancer research as it poses a critical challenge to designing effective treatment regimens in the era of personalized medicine [171]. While it is tempting to suggest that next-generation FASN inhibitors broadly affecting the lipogenic phenotype of tumor tissue should be less vulnerable to intra-tumor heterogeneity, it should be considered that the lipogenic features of the most abundant cell type might not necessarily predict the lipogenic status of heterogeneous cell populations and, by extension, their intrinsic degree of responsiveness or primary (innate) resistance to FASN inhibitors. With the technological advances in recent years, investigators are now characterizing both genetic and epigenetic sources of intra-tumor heterogeneity. Unfortunately, little is known about the metabolic origins and causes of phenotypic heterogeneity, especially on the role that endogenous lipogenesis might play on the generation and maintenance of heritable phenotypes that would serve as substrates for clonal selection and tumor evolution. Indeed, the old notion that cancer metabolism is a single entity that differs from normal cell metabolism no longer holds true as we are learning that a single model of altered tumor metabolism or metabolic map cannot describe all the metabolic changes that support cancer growth and progression [172–175]. Therefore, we can predict that cell-autonomous and non-cell autonomous regulation of tumor metabolic plasticity, which not only imparts heterogeneity in the metabolic dependencies of tumor cells but also allows tumor tissues to adapt and grow in hostile microenvironments via metabolic symbiosis [176–179], will rapidly emerge as an important clinical dimension that should be anticipated when developing FASN inhibitor-based approaches. A better understanding of how intratumoral regional variation in metabolically challenging oxygen environments including low-oxygen and lipid-depleted conditions might contribute to tumor metabolic heterogeneity would enable the development and optimization of new therapeutic strategies aimed to target FASN-related endogenous lipogenesis. In this regard, the recent description of the ability of the acetyl-CoA synthetase 2 (ACSS2) to impart competitive growth advantages under conditions of metabolic stress by enhancing the ability of breast cancer cells to use acetate as an additional lipogenic substrate when other carbon sources cannot be used to sustain lipid biomass production [180] has potential to clinically develop acetate-based PET tracers for detecting when tumors might become refractory to FASN-targeted therapy and supports new therapeutic approaches combining FASNinhibs with currently being explored ACSS2 inhibitors [180,181].

8.2. Acquired resistance to next-generation FASN inhibitors

The widely accepted view that many genetic lesions important for cancer all converge to promote proliferative metabolism in cancer cells [18–20] has led to the equivocal suggestion that targeting cancer metabolism should be a simpler approach than targeting numerous mutated gene products. In such a scenario, therapeutic interventions with metabolic inhibitor-based therapies, including FASN blockers, are expected to be less susceptible to acquired resistance; however, as for conventional chemotherapeutics and modern targeted agents, one might predict that intratumoral metabolic heterogeneity

will underlie incomplete responses, the development of acquired resistance, and disease relapse to treatment with next-generation FASN inhibitors. Given the intrinsic metabolic flexibility of cancer cells, exclusively targeting specific metabolic pathways such as FASN-driven endogenous lipogenesis might be just as complicated as targeting somatic mutations, if not more so [182,183].

With the sole exception of one *in vitro* finding that nuclear factor-kappa B might mediate a protective response in lung cancer cells treated with the FASN inhibitor C93 [184], no study has assessed the possibility that specific pharmacological targeting of FASN activity may result in unforeseeable acquired resistance in initially responsive breast carcinomas. Preclinical models of breast cancer cells adapted to grow for several months in the presence of metformin, which negatively regulates the expression of lipogenic markers, imposed a strong selective pressure for the emergence of new breast cancer cellular states [185]. Thus, while refractoriness to the anti-lipogenic actions of metformin dramatically limited breast cancer cell growth, it conspicuously increased the potential of metastatic dissemination by amplifying several pro-migratory and stemness inputs via the activation of a significant number of proteases and positive regulators of epithelial-to-mesenchymal transition (EMT) [185,186]. Accordingly, recent studies in lung carcinoma cells have confirmed that suppressing endogenous lipogenesis might be an essential metabolic component of EMT required for successful establishment of distant metastases [187]. However, because FASN inhibition has been found to prevent metastasis-related phenomena including pseudopodia formation and cellular adhesion, migration, and invasion [188,189], future studies should assess whether FASN activity, while co-opted as a metabolic component of the cell motility machinery in breast cancer metastatic progression [190], might have the troublesome consequence of increasing long-term risk of EMT-related cell migrations and metastases in breast cancer patients treated with FASNinhibs.

8.3. FASN inhibitors and breast cancer stem cells

FASN expression has been found to be hyperactivated in proliferating fetal tissues [191] and also in induced pluripotent stem cells [192], suggesting that re-activation of FASN-catalyzed endogenous FA synthesis might participate in the reversion to less-differentiated embryonic states such as those characterizing the so-called cancer stem cells (CSCs). Since CSCs can survive treatment with hormones, radiation, chemotherapeutic agents, and molecularly targeted drugs, the capacity of CSCs for autorenewal and differentiation might ultimately be responsible for the clinical failure of current oncology therapies [193–196]. CSCs appear to exhibit unique metabolic features that are required not only for supporting specific CSC bioenergetic/biosynthetic demands, but also for epigenetically sustaining their operational properties, that is, self-renewal, tumor-initiation, and plasticity [197–200]. While the metabolic infrastructure of CSCs in breast carcinomas remains controversial and understudied, it appears that CSC-like cells express significantly higher levels of several lipogenic enzymes including FASN [201–205]. The (-)-C75 enantiomer of C75, which specifically inactivates FASN without affecting

carnitine palmitoyltransferase 1-related food consumption [206], was found to drastically suppress the ability of CSC-like cellular states to survive and proliferate as floating spherical colonies under anchorage-independent, non-differentiating conditions, an *in vitro* proxy of self-renewal and tumor-initiating potential exclusively possessed by CSCs [55]. The natural polyphenolic compound resveratrol has also been shown to efficiently target CSC-like cells via suppression of lipogenesis by modulating FASN [201]. Future studies should examine whether FASN represents a powerful, but hitherto largely unexplored, target to eliminate treatment-refractory CSCs.

8.4. FASN inhibitors and normalized breast epithelial differentiation

Beyond conferring survival advantages to CSC-like states in preinvasive breast cancer lesions such as ductal carcinoma *in situ* [199], FASN-driven maintenance of an undifferentiated state in stem-like cells might play unexpected roles in dictating breast tissue architecture, thus opening the way for the use of next-generation FASN inhibitors as chemopreventative agents in early stages of breast cancer development. We recently evaluated whether the correction of FASN-catalyzed exacerbated endogenous lipogenesis might be sufficient to stably revert the malignant phenotype during breast cancer development [205]. The activation status of FASN appeared to dictate the degree of refractoriness/responsiveness of breast epithelial cells to differentiation/dedifferentiation phenomena, and therefore, their intrinsic susceptibility to the epigenetic rewiring required for the activation of a pathological differentiation program of aberrant stemness. From a Waddingtonian perspective [199], the correction of exacerbated lipogenesis might cause a distortion of the epigenetic landscape, allowing cells in a tissue organization attractor that encodes a proliferative, CSC-like undifferentiated phenotype, to suddenly re-acquire a normalized phenotype by placing them in a self-stabilizing attractor encoding a more quiescent, differentiated epithelial-like tissue phenotype [205]. An ever-growing body of evidence increasingly recognizes the exquisite responsiveness of the epigenetic regulatory machinery to metabolic cues [207–209]. Because most of the chromatin modifiers employ metabolic products as substrates or cofactors while chromatin modifications such as acetylation and methylation are known to depend on the functionality of certain metabolic fluxes including those involving endogenous lipogenesis [210,211], the ability of FASN signaling to regulate breast tissue architecture and terminal epithelial differentiation in a dominant manner over the malignant phenotype of tumors possessing multiple cancer-driving genetic lesions can provide not only new FASN inhibitor-based therapeutic options to chronically restrain the life-threatening potential of invasive carcinomas, but can also uncover a poorly understood epigenetic dimension of FASN signaling that is likely to be a fertile area for future investigation.

9. Expert opinion

FASN has been considered an attractive target for breast cancer therapy in the last decade. Unfortunately, most preclinical studies have failed to efficiently move from the basic

science-discovery aspects of FASN inhibition into the clinical arena. With the recent demonstration of target engagement and early signs of clinical activity with the first orally available, selective, potent, and reversible inhibitor of FASN in breast cancer patients, we can expect Big pharma to revitalize their interest in endogenous lipogenesis as a well-credentialed target for oncology drug development. We anticipate that additional FASN inhibitors will be integrated into a mechanistically richer and expanding pipeline of targeted drugs based on an ever-growing understanding of the FASN biology-breast cancer association. In the forthcoming clinical research scenario, FASN inhibitor-diagnostic co-development programs should be rapidly implemented to identify decisive stratifications factors, having the potential to be important tools for clinicians in relation to: (1) the identification of breast cancer subtypes most likely to benefit from FASN inhibitors (e.g. HER2-overexpressing [55,141,142] and triple-negative [116,136] breast carcinomas); and (2) monitoring the response to FASN inhibition to achieve improved effectiveness and safety. Such companion diagnostic strategies, which should have a high degree of analytical validity before they can be released for routine clinical usage, will be critical to accelerate the development of next generation FASN inhibitors. Beyond monitoring tumor FA biosynthesis by functional PET-imaging using $[^{11}C]$-acetate [212,213] to assess therapeutic responses to drugs directly or indirectly interfering with FASN-catalyzed FA synthesis, other label-free imaging techniques (e.g. Raman spectroscopy or imaging mass spectrometry) might provide novel pharmacodynamic biomarkers to determine the actual engagement of breast carcinomas to *de novo* synthesis of FAs in response to FASN inhibitors [214]. Beyond circulating serum/extracellular FASN as a dynamic marker of metabolic stress/normalization, we could incorporate multi-metabolite panels based on the identification and validation of metabolomic/fluxomic fingerprints related to the efficacy and safety of FASN inhibitors. The exploration of the circulating exo-metabolome to monitor, in real-time, such biomarker/surrogate endpoints of FASN inhibitor efficacy in liquid biopsies might optimize the development and accelerate a better design of FASN inhibitor-based personalized breast cancer therapies.

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