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EDITORIAL

^{18}F-fluoro-2-deoxy-d-glucose (FDG) uptake. What are we looking at?

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"Mr. Holmes, they were the footprints of a gigantic hound!"
Arthur Conan Doyle, The Hound of the Baskervilles

Introduction

In the last decade, the possibility to map glucose consumption with 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) imaging profoundly modified the daily activity of most nuclear medicine services. Despite the notion that glucose metabolism is probably the most elementary feature of life and is shared by all living cells, the diagnostic accuracy and the predictive power of PET/CT imaging, coupled with its user-friendly procedure, configured this technique as the clinical revenue for the link between glucose consumption and cancer aggressiveness. This concept was anticipated by Otto Warburg who described, already in 1924, the acceleration of glycolytic flux and its independence of oxygen tension in cancer [1]. The unprecedented clinical success of FDG imaging even contaminated the research activity of basic science investigators, as documented by the PubMed database reported in Fig. 1 that shows how (and when) the number of experimental studies containing the terms "Warburg effect" grew soon after the expansion of clinical studies containing the term "FDG."

In the era of evidence-based medicine, the clinical application of FDG imaging is only justified by the documented benefit it can provide on patient management. Nevertheless, as for any other technique, its scientific background should not be limited to a detailed competence about the clinical indications for its use. Rather, a thorough understanding of the mechanisms underlying its diagnostic power is the mandatory prerequisite to deepen our interpretation of its informative content as well as to optimize its clinical application and to improve our comprehension of disease features.

So far, the link between FDG uptake and overall glucose consumption is almost universally accepted. Nevertheless, a series of experimental pieces of evidence and theoretical considerations suggest that this interpretation might be only partially correct. Discussing this literature, here we aim to provide a different and challenging description of the mechanism regulating FDG retention and distribution within the cell. The emerging picture suggests that tracer uptake is only loosely linked to glycolytic flux and might rather reflect the metabolic adaptations to the high NADPH requirements, selectively featuring cells with high proliferating activity or exposed to an intense redox stress.

Current model of FDG uptake

The current interpretation of FDG imaging derives from the tight and local connection between glucose consumption and ^{14}C-2-deoxyglucose (2DG) uptake, described in the seminal work by Sokoloff et al. [2] in the brain of albino rats. Phelps and coworkers extended this kinetic model to FDG [3] and thus to humans, allowing the subsequent method optimization for diagnosis and staging of solid cancers [4], neurodegenerative disorders [5], and inflammatory diseases [6], as well as

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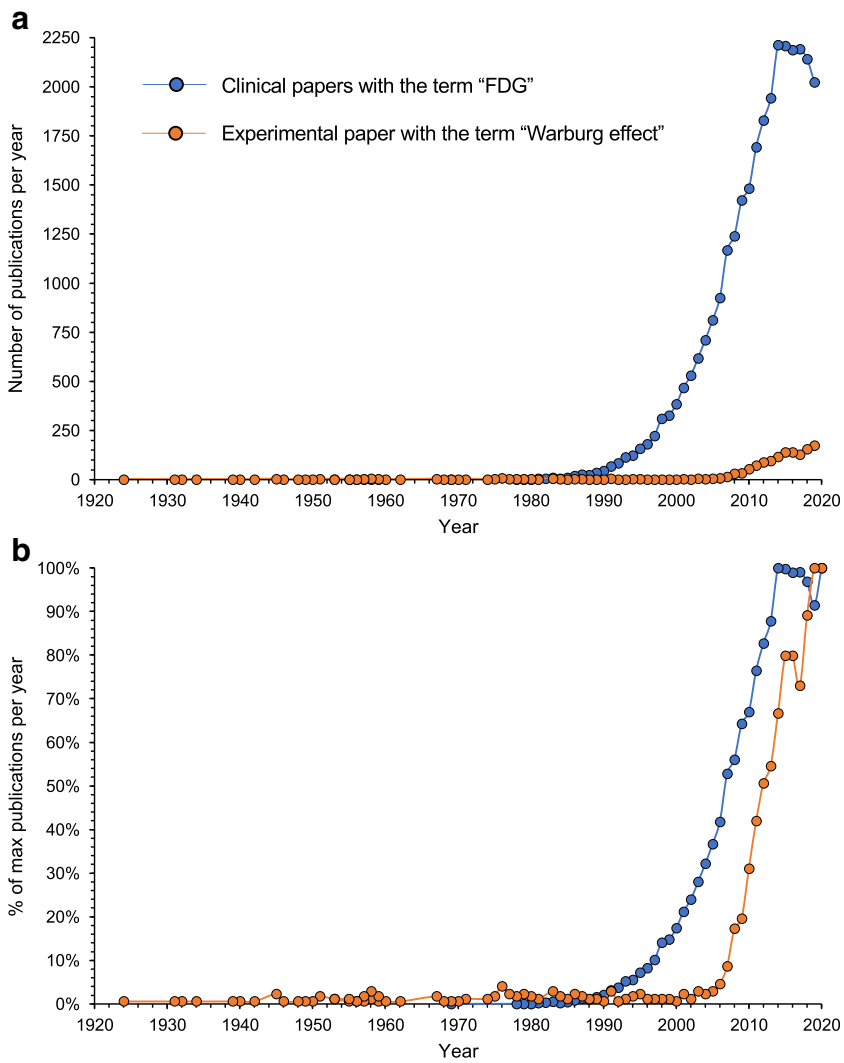

Fig. 1 Panel **a** shows the number of papers (Y axis) published in each year since 1920 (X axis). Blue line indicates the published papers containing the term "FDG" available on PubMed database and limited to humans. The orange line displays the number of papers including the words "Warburg effect" and not related to humans. Panel **b** shows the same data expressed as percent of the maximal number of papers that occurred in year 2015 for "FDG" and 2020 for "Warburg effect"

for the detection of residual viability in patients with previous myocardial infarction and left ventricular dysfunction [7].

Its theoretical basis relies on the assumption that both 2DG and FDG compete with glucose for GLUT-facilitated transmembrane transport and hexokinase-catalyzed phosphorylation [8]. This competition implies that the intracellular production of either 2DG-6-phosphate (6P) or FDG-6P is directly related to the generation rate of glucose-6P (G-6P). Both phosphorylated analogues are false substrates for downstream enzymes, channeling G6P to glycolysis or pentose phosphate pathway (PPP) [9] and, thus, accumulate within the cytosol as dead-end metabolites.

According to this model, the uptake of FDG- or 2DG-vehiculated radioactivity does not permit to trace the complex metabolic fate of native glucose through glycolysis, PPP, and the many other pathways that interconnect each other in intermediary metabolism. Yet, this biochemical arrest provides two main advantages. On one side, it permits to investigate glucose metabolism in virtually all tissues, regardless their normal or pathological nature. On the other side, it facilitates the analysis of tracer time-concentration curves in arterial blood and tissues to estimate the rate of FDG influx and phosphorylation. Obtained rate constants can be extended to glucose, once normalized for substrate availability in the bloodstream. With few exceptions, the metabolic pattern can be assumed to be stable during FDG uptake time, particularly when a tracer is injected under fasting conditions. Glucose assets of investigated tissues can be thus considered invariant, permitting to consider glucose influx/phosphorylation as equal to glucose consumption.

The further advantage of FDG imaging is that it avoids the need for dynamic scan procedures to directly estimate the rate constants of tracer exchanges between blood and cells, at least for tissues characterized by a negligible tracer loss. Indeed, this complex estimation can be surrogated by mapping FDG concentration when virtually all tracer molecules have been cleared from the blood and sequestered within the various organs. At this time, the standardized uptake value (SUV)

can be calculated as the ratio between local tracer concentration and the expected average body concentration of the injected dose [10]. Assuming comparable metabolic patterns in fasting humans, the single "static" image-acquisition permits to compare the glucose metabolic rates of various tissues or lesions among different patients, using a procedure compatible with the high patient-throughput requested to the clinical nuclear medicine labs.

Experimental challenges to the Sokoloff model

Despite its almost universal acceptance, the strict link between glucose consumption and uptake is challenged by a series of experimental studies. According to the classical model, cells should accumulate 2DG and FDG in their phosphorylated form 2DG-6P and FDG-6P. However, this hypothesis has barely (if ever) been confirmed.

Looking at the "father compound" 2DG, a number of studies documented the presence of a significant metabolism of 2DG-6P characterized by its oxidation and subsequent decarboxylation through the PPP [11–13]. This processing profoundly hampers the estimation of the rate of 2DG phosphorylation to 2DG-6P by experiments requiring the chemical methods, due to the interference of still unidentified metabolites converted back to 2DG by the hexose extraction with perchloric acid [14, 15].

A similar consideration also applies to FDG, whose metabolic fate has been intensively investigated after the introduction of nuclear magnetic resonance spectroscopy (NMRS) and its capability to estimate fluorinated metabolites after injection of FDG labeled with the stable isotope ^{19}F (^{19}F-FDG). Using this approach, different studies reported a progressive enrichment of rat brain with at least two PPP-related metabolites after a large loading dose of ^{19}F-FDG: 2-F-2-deoxy-d-phosphogluconolactone and 2-F-2-deoxy-6-phosphogluconate [16–18], whose abundance progressively increases in the first hour after tracer injection [19]. Both observed metabolites are intermediates of PPP, whose relevance in FDG-6P processing has been subsequently confirmed by the evidence that, 2 days after administration, ^{19}F-labeled metabolites are linked to ribose-containing nucleotides, at least in tumors and myocardium [20].

As a final consideration, at least two studies verified to what degree ^{19}F-FDG-6P processing is tissue-dependent. Southworth and colleagues demonstrated that cell enrichment of downstream metabolites is highest in the brain (45%), intermediate in the myocardium (29%) or liver (22%), and lowest in the kidney (17%), 90 min after tracer injection [21]. On the other hand, Shinohara et al. reported that FDG6P conversion is dependent upon tissue function since it is impaired by anesthesia in the brain but not in the heart or skeletal muscle [22].

Altogether, these findings challenge the basic assumption of the Sokoloff model, i.e., the terminal-metabolite nature of 2DG-6P and FDG-6P. Actually, it is well recognized that both phosphorylated glucose-analogues are false substrates for the two enzymes downstream hexokinases: G-6P-isomerase and G-6P-dehydrogenase (G6PD) [8, 9]. Nevertheless, both 2DG-6P and FDG-6P can be, and actually are, processed. This metabolism might be completely independent of glycolysis and cytosolic PPP. Its selective activation or inhibition might thus invalidate the commonly assumed equivalence of radioactivity retention and overall glucose consumption. Although its reaction sequence is still largely unknown, this pathway (or pathways) should be somewhat related to PPP, as suggested by the presence of fluorinated intermediates of its oxidative and non-oxidative branches [11–22].

Theoretical shortfalls of the Sokoloff model

The experimental mismatches are further confirmed by the theoretical implications of the two main assumptions underlying the classical model. These can be synthesized as (1) the competition between FDG and glucose for transmembrane transport and phosphorylation is constant and known and (2) the accumulated radioactivity cannot be lost.

The former assumption accounts for the notion that the FDG kinetic parameters are not identical to the glucose ones. This means that transmembrane transport through the different GLUTs and phosphorylation by one of the four hexokinases can have different rates for glucose with respect to FDG. These same considerations have been extended to the hydrolysis reaction, catalyzed by G6P-phosphatase (G6Pase), despite its very low rate in the majority of studied tissues. Accounting for the different Michaelis-Menten kinetic parameters of all involved factors requires considering a proportionality factor that had been formally defined as lumped constant (LC), since the first Sokoloff study [2, 23]. LC is usually estimated by the simultaneous measurement of both FDG (or 2DG) uptake rate and glucose consumption. This approach indeed documented that LC varies in different tissues, according to the relative abundance of the different GLUTs and hexokinases isoforms. Nevertheless, this variability has been duplicated even by studies focused on the same tissue. As a matter of fact, brain LC has been found to vary from 0.52 [24] up to 1.24 [25], resulting in an unacceptable uncertainty (>100%) in the estimation of glucose consumption.

Although a number of methodological considerations have been claimed, this disappointing unpredictability has not been fully explained, and, more importantly, it is dependent upon the experimental condition. Indeed, LC has been found to rapidly increase with decreasing glucose availability both in cultured cancer cells (from 0.7 to 1.22) [26] and in brains of living animals (from 0.41 to 1.24) [25]. Similarly, the ratio between FDG uptake and glucose consumption (and thus the

LC value) has been found to instantaneously increase during neuronal activation [27]. These shifts are extremely fast, occurring in a time range of seconds and thus are relatively unlikely to reflect corresponding changes in the type of active GLUTs or hexokinases as well as of G6Pase abundance. Indeed, modifying gene expression would obviously require a much longer time, particularly in tissues (as cancer or brain) with scarce or absent GLUT4 expression. Again, these findings thus challenge the validity of the conventional interpretation of FDG kinetic and suggest that the selective activation of the FDG6P processing pathway might contribute to LC determination.

The latter consideration, i.e., the irreversible nature of FDG accumulation, relies on the notion that the electrically charged FDG6P cannot cross the cell membrane and can be released back into the bloodstream only after its hydrolyzation by G6Pase. When the method was introduced in the late 1970s, this enzymatic activity had been only documented in tissues dedicated to the preservation of serum glucose level during fasting periods: liver, gut, and kidneys. Nevertheless, Sokoloff et al. observed a measurable 2DG washout from rat brain, whose radioactivity content decreased with a half-life of 7.7 ± 1.6 and 9.7 ± 2.6 h in gray and white matter, respectively [2]. Similarly, a slow, yet measurable, tracer release has been subsequently confirmed from several cancers, suggesting the possible presence of a G6Pase function in tissues of different origin [28, 29]. This slow washout has been largely disregarded according to the consideration of its negligible interference on imaging performed within 1 h after tracer injection. Yet, its measurability inevitably implies the presence of a FDG6P hydrolyzation in all investigated tissues.

This puzzle has been explained by the documentation, provided by the literature on glycogen storage diseases, that G6Pase activity is actually warranted by two different enzymes with similar k_m: the "original" G6Pase-α restricted to liver-kidney-intestine and the "new" G6Pase-β (or G6PC3) that is ubiquitously expressed in all tissues [30] and in several cancer types, including the uterus, lung, glioblastoma [31], breast, and colon [32].

From a practical point of view, G6Pase-β thus explains the FDG clearance from nervous and cancer tissues without affecting its relevance on the estimation of tracer uptake. However, this reasoning intrinsically represents an approximation since both G6Pase isoforms are embedded in the endoplasmic reticulum (ER) membrane [30, 33–35] with their catalytic function confined within the ER lumen. According to this confinement, G-6P de-phosphorylation requires the presence of a specific pump able to carrier FDG-6P through the reticular membrane. This transporter has been identified as the ATP-dependent G6P-translocase (G6PT) (SLC37A4) [36, 37] that participates in the functional complex G6PT/G6Pase to allow the hydrolyzation of G6P, while glucose back-diffusion to the cytosol is warranted by the high and variegate GLUT asset of ER membrane [38]. This same functional complex also processes FDG-6P, and, indeed, tracer washout has been found to reflect G6PT expression more than G6Pase activity [39].

Although the mechanisms governing G-6P/glucose exchange between cytosol and ER remain largely undefined, the reticular confinement of G6Pase configures the ER as the FDG escape gate and retains profound implications on tracer accumulation kinetics. Indeed, the activity of cytosolic glucose processing pathways maintains relatively stable and low G-6P concentrations in the cytosol, differently from the "non-processable" FDG6P that accumulates. This feature would imply a progressive increase of tracer transfer to the ER lumen and thus a progressive acceleration of radioactivity washout, profoundly modifying our interpretation of FDG kinetics. Scussolini et al. approached this issue using a mathematical analysis of FDG time-concentration curves in cultured cancer cells [40]. Obtained results documented that tracer kinetics is compatible with the sequestration of G6Pase in the ER lumen and configures hexokinase activity to levels much closer to its expected theoretical value with respect to the Sokoloff model. Obviously, this design configures the ER as the preferential site of FDG accumulation but requires the presence of a local mechanism able to prevent G6Pase catalysis and to channel FDG6P toward its final destination.

This analytical conclusion is confirmed by the confocal microscopy evidence of a progressive co-localization of the signal vehiculated by the fluorescent FDG analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) [41] with the ER probe glibenclamide. Intriguingly, this evaluation permitted to confirm that ER accumulation of both FDG and 2-NBDG is actually modulated by glucose concentration in the culture medium, thus providing a possible explanation of the previously quoted rapid shift in LC in response to nutrient availability [40].

The ER as escape gate and accumulation site of FDG radioactivity

Altogether, experimental findings and theoretical considerations indicate that FDG accumulation might at least partially reflect the activation degree of a still undefined glucose processing pathway that should be related, but not identical, to the PPP and located within the ER.

Although not usually considered in studies focused on FDG kinetics, a processing machinery accounting for both requisites has been actually recognized since a long time. Indeed, some years after the introduction of the FDG method, Bublitz et al. reported that liver microsomes, as epitomes of hepatocytes ER, actually contain a full asset of PPP enzymes [42], while subsequent literature extended this function to virtually all tissues [42]. This reticular pathway is triggered by a specific enzyme, called hexose-6P-dehydrogenase (H6PD),

whose features seem particularly sound for cell retention of FDG-vehiculated radioactivity. Indeed, differently from its cytosolic – sex-linked – alter ego G6PD, the autosomic H6PD can dehydrogenate a large variety of phosphorylated and free hexoses [43–45] including FDG-6P and 2DG-6P. Moreover, its reticular confinement configures H6PD catalytic function as competing with G6Pase and thus able to limit the hydrolysis and the back-flux of phosphorylated hexoses.

The H6PD-triggered glucose metabolism seems thus to represent a metabolic machinery fitting most – if not all – theoretical determinants of FDG and 2DG kinetics. Nevertheless, the role of ER-PPP in this setting drew scarce attention, most likely because it has been usually considered a very low-rate metabolism processing small amounts of G6P to feed the activation of steroid hormones by the NADPH-dependent reticular enzyme $^{11}\beta$-hydroxysteroid-dehydrogenase [44]. More recently, this concept has been challenged by a series of observations. In particular, Cossu et al. used a NMRS approach to verify the metabolic pattern of human cell lines derived from breast and lung cancers after the inhibition of H6PD or G6PD expression by short-interfering RNA (siRNA) [45]. Silencing either enzyme caused a superimposable decrease in PPP activity as documented by the fall in pathway intermediates and NADPH/NADP ratio that, in turn, was followed by a comparable increase in the cell content of reactive oxygen species. Accordingly, this metabolomic evaluation indicates that H6PD processes comparable G-6P amounts with respect to G6PD. More importantly, it also suggests that the reticular PPP might be completely autonomous and active on G6P pools not shared by the cytosolic one.

Based on these considerations, we hypothesized that FDG uptake might actually reflect the degree of activation of H6PD-triggered ER-PPP. In murine colon and breast cancer cell lines [32], siRNA-induced silencing of H6PD expression profoundly decreased FDG uptake despite a marked acceleration of lactate release and thus of glycolytic flux in both cell types. Again, the role of reticular PPP in FDG accumulation rate was confirmed the ER localization of the fluorescent 2-NBDG that, at confocal microscopy, was markedly inhibited by H6PD-siRNA.

This observation was also reproduced in normal tissues. Studying the response of glucose consumption and FDG uptake to the prolonged treatment with high doses of metformin in the normal mouse brain, Cossu et al. documented a profound decrease in FDG uptake [46]. This effect was reproduced ex vivo and mismatched the marked increase in glucose consumption induced by the drug-related inhibition of respiratory activity. The consequent steep fall in LC was not explained by any change in expression or activity of its theoretical determinants (GLUTs, hexokinases, or G6Pase), while it agreed with the metformin-induced inhibition of H6PD function. A similar mismatch also characterized the divergent metabolic pattern of astrocytes and neurons: the high glycolytic flux of the former was associated with a very low FDG uptake that was instead high, despite the relatively lower glucose consumption, in neurons. Again, this difference reproduced the gradient in H6PD activity between the two cell types paralleled by a divergent rate of 2-NBDG accumulation within the ER.

Besides cancer and brain, the most common target of FDG imaging, this same concept has been also extended to other districts. Marini et al. investigated the mechanisms underlying the increased FDG uptake and its prognostic power in patients with amyotrophic lateral sclerosis [47, 48] by measuring tracer retention and glycolytic flux in the hind limb muscles of $SOD1^{G93A}$ mice [49]. The increased FDG uptake was reproduced in this disease model despite an invariant glycolytic flux. By contrast, it was associated with an evident activation of H6PD catalytic function as a possible response to the significant redox stress typical of this validated model of motor neuron disease and, at least partially, caused by an alteration of mitochondrial structure, networking, and connection with the ER.

These same considerations were finally extended to the myocardium. Actually, cardiac FDG uptake has been found to be increased in patients treated with doxorubicin because of Hodgkin lymphoma [50]. This same finding was reproduced in experimental mice in which the anthracycline-related oxidative damage expectedly increased the myocardial content of reactive oxygen species [51]. This redox stress was associated with an increased tracer retention whose degree was related to the activation of H6PD catalytic function, suggesting a possible demand for ER NADPH equivalents to feed local antioxidant responses. Again, ex vivo simultaneous measurement of cardiac tracer uptake and glucose consumption documented a marked increase in LC that was not related to any change in its theoretical determinant, thus confirming the elusive link between FDG retention and glucose consumption [25–27].

FDG uptake as a potential index of ER-PPP activation in cancer and inflammation

All reported considerations profoundly challenge the currently assumed equivalence of FDG uptake and glucose consumption, indicating a relevant role for H6PD-triggered metabolism in tracer accumulation and retention. This hypothesis thus implies that ER-PPP activation should be involved in all those conditions in which FDG imaging has been proven to characterize disease presence and aggressiveness. This assumption has not been confirmed by an adequate literature. Yet, it fits with a series of considerations particularly relevant in the progression of cancer and inflammatory disorders.

Actually, the already quoted “Warburg effect” is usually attributed to the ATP need of growing tumors. However, this concept largely represents an oversimplification. Indeed, cell proliferation asks for many different building

blocks whose setup requires a series of factors beyond the adequate energy asset. As an example, the synthesis of cell membrane fatty acids consumes 35 times more glucose to feed NADPH-derived electrons than to warrant the needed ATP moieties [52].

In mammalian cells, the main pathway dedicated to NADP reduction to NADPH is PPP [53], whose dual step nature of reactions sequence permits cells to adapt their metabolic pattern to endogenous and exogenous signals [54, 55]. Enhanced redox stress selectively accelerates the first oxidative phase as to feed the NADPH- and glutathione-dependent antioxidant responses. By contrast, PPP acceleration can extend to the non-oxidative phase when the high proliferating activity asks to combine bio-reductive syntheses and thus high NADPH levels, with large amounts of D-ribose-5P for the production of RNA, DNA, nucleosides, ATP, coenzyme A, and other coenzymes such as NADH, $FADH_2$, and NADPH [56]. Since its discovery by Warburg in 1931 [57], most studies equated the PPP relevance on cancer growth to the activity of G6PD [54, 55]. Nevertheless, the role of this enzyme in this setting is markedly less obvious, since even severe G6PD deficiency (down to <1% of normal activity) does not decrease cancer incidence [58, 59] and can even increase mortality for several cancer types [60].

The previously quoted considerations actually indicate an unexpected relevance for the ER-PPP, in line with the evidence of an enhanced H6PD expression in several types of malignancies (https://www.proteinatlas.org/ENSG00000049239-H6PD/pathology). Indeed, silencing H6PD or G6PD not only resulted in comparable consequences on the metabolic and redox profile [45]. Rather, they also caused a similar cell cycle arrest at the G1/S phase transition eventually resulting in an inhibition of cell proliferating activity [32, 45, 61–63]. Similarly, Tsachaki et al. reported that inhibiting H6PD expression simultaneously impairs the proliferation rate and migratory capacity of breast cancer cells [61]. Finally, Ji and coworkers extended this finding to gallbladder cancer, documenting that the expected response to gene silencing matched an enhanced cancer growth in vitro and in vivo by H6PD overexpression [64].

A similar consideration also applies to the meaning of FDG imaging in inflammation. Besides, the specific H6PD role in activating corticosteroid hormones [44], the role of ER PPP in this setting is further confirmed by the response of tumor-associated macrophages to the selective inhibitor of G6PT chlorogenic acid. Preventing G6P access to ER lumen, and thus to H6PD processing, markedly favored macrophage re-polarization toward the pro-inflammatory M1 phenotype [65]. In agreement with this concept, our preliminary data reported in Fig. 2 show that ER accumulation of 2-NBDG is impaired by 24-h incubation with 70 μM concentration of chlorogenic acid in bone marrow-derived macrophages. A similar observation is reproduced by the same cell types harvested from $H6PD^{-/-}$ mice that showed a marked decrease in the reticular location of 2-NBDG vehiculated signal.

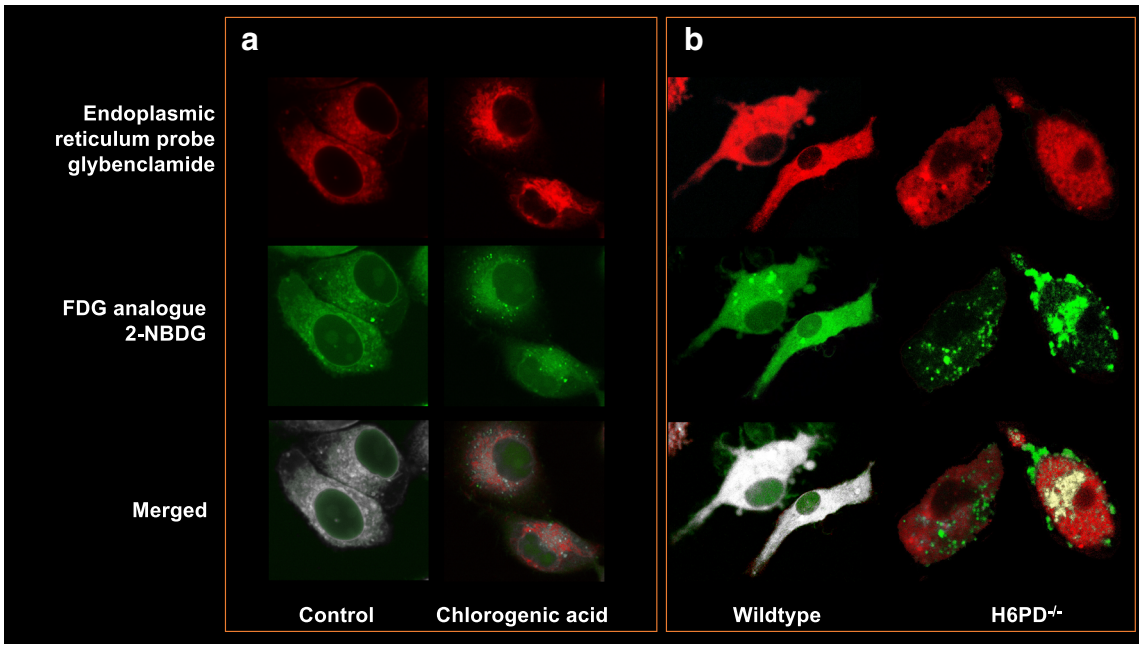

Fig. 2 Confocal microscopy images of breast cancer cells MDA-MB231 (panel **a**) and bone marrow-derived macrophages (panel **b**). The top row displays the staining with the ER probe glibenclamide; middle row, 2-NBDG accumulation; and bottom row, the colocalization data obtained with Costes method [66]. In MDA-MB231, inhibition of G6PT effectively inhibited 2NBDG accumulation within the ER. The same ER accumulation was markedly impaired in bone marrow-derived macrophages harvested from H6PD−/− mice that were provided by prof. Lavery as a courtesy

Conclusions

In conclusion, the literature commented in the present editorial commentary indicates that 2DG, FDG, and 2-NBDG do not accumulate as "inert phosphorylated glucose analogues." Accordingly, the retention of these tracers cannot be considered an index of glucose consumption and, even less, of local glycolytic flux. It rather most likely reflects the rate of a still undefined ER metabolic machinery triggered by the omnivore enzyme H6PD, largely independent of cytosolic glucose metabolism, "geographically" confined within the ER and dedicated to the local control of NADPH- and PPP-derived metabolites.

This paradigm shift applies to both normal and diseased tissues. On the clinical ground, its most evident revenue best applies to cancer patients, in whom it might explain the high diagnostic and prognostic power of FDG retention, without attributing this clinical value to its link with glucose consumption and thus with the most elementary feature of life, shared by all living cells. From the basic science perspective, coupling the high clinical performance of FDG uptake and its evident link with ER-PPP potentially configures the same ER as a pivotal determinant of cancer aggressiveness through mechanisms that definitely need further investigations.

Abbreviations (FDG), ^{18}F-fluoro-2-deoxy-d-glucose; (2DG), 2-deoxyglucose; (2-NBDG), 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose; (PPP), pentose-phosphate pathway; (ER), endoplasmic reticulum; (H6PD), hexose-6-phosphate-dehydrogenase; (G6PD), glucose-6-phosphate-dehydrogenase; (G6Pase), glucose-6-phosphate phosphatase

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Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Ethics approval Institutional review board approval was not required because the paper is an editorial.

Informed consent Not applicable.

Conflict of interest The authors declare no competing interests.

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