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Expression of the hypoxia-inducible monocarboxylate transporter MCT4 is increased in triple negative breast cancer and correlates independently with clinical outcome

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ABSTRACT

Background: ^{18}Fluor-deoxy-glucose PET-scanning of glycolytic metabolism is being used for staging in many tumors however its impact on prognosis has never been studied in breast cancer.
Methods: Glycolytic and hypoxic markers: glucose transporter (GLUT1), carbonic anhydrase IX (CAIX), monocarboxylate transporter 1 and 4 (MCT1, 4), MCT accessory protein basigin and lactate-dehydrogenase A (LDH-A) were assessed by immunohistochemistry in two cohorts of breast cancer comprising 643 node-negative and 127 triple negative breast cancers (TNBC) respectively.
Results: In the 643 node-negative breast tumor cohort with a median follow-up of 124 months, TNBC were the most glycolytic (≈70%), followed by Her-2 (≈50%) and RH-positive cancers (≈30%). Tumoral MCT4 staining (without stromal staining) was a strong independent prognostic factor for metastasis-free survival (HR = 0.47, $P = 0.02$) and overall-survival (HR = 0.38, $P = 0.002$). These results were confirmed in the independent cohort of 127 cancer patients.
Conclusion: Glycolytic markers are expressed in all breast tumors with highest expression occurring in TNBC. MCT4, the hypoxia-inducible lactate/H^+ symporter demonstrated the strongest deleterious impact on survival. We propose that MCT4 serves as a new prognostic factor in node-negative breast cancer and can perhaps act soon as a theranostic factor considering the current pharmacological development of MCT4 inhibitors.

1. Introduction

Cancers are defined by unlimited proliferation of tumoural cells and consequently require a constant supply of large amounts of nutrients such as glucose, lipids and amino acids [1,2]. GLUT-1 is particularly expressed in aggressive and often hypoxic glycolytic tumors. High expression of GLUT-1 correlates directly with the ^{18}Fluor-deoxy-glucose (^{18}FDG) uptake during PET-scanning in patients (pts). Compared to non-malignant tissues, cancers have high rates of glucose uptake and consumption through glycolysis.

Mammalian cells produce ATP either via converting glucose to pyruvate which can be metabolized in the mitochondria through the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS/respiration), or reduced in the cytoplasm into lactic acid through the fermentative/anaerobic glycolysis *via* lactic dehydrogenase (LDHA). However, Otto Warburg demonstrated that tumor cells, preferentially metabolize pyruvate into lactic acid regardless of the oxygen concentration [3] which leads to increase rates of glycolysis and associated uptake of glucose. Carbonic and lactic acids are metabolic waste products that could compromise tumor growth and cellular viability if they are not rapidly exported out of the metabolic active cells. Consequently, rapidly growing tumor

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cells overexpress proteins that allow extrusion of these acids, such as carbonic anhydrases (CAs) [4] and monocarboxylate transporters (MCTs) [5] which contribute to the homeostasis of intracellular pH (pH_i) [6]. Promising therapeutic strategies targeting the strong glucose addiction of cancer cells have consequently emerged [7–11] even if no drugs are yet currently approved in this field.

We decided to explore the exacerbated glucose metabolism. Interestingly ^{18}FDG uptake was reported to be higher in triple negative breast cancers (TNBC) subgroup with a 100% sensitivity and a much higher standard uptake value (SUV) in comparison with hormone receptor or Her-2 positive tumors [12,13]. Glycolytic and often hypoxia-induced markers [14,15] contributing to this increased ^{18}FDG uptake could therefore correlate with survival and provide new independent prognostic markers to identify patients who need adjuvant chemotherapy especially in node negative cases. The impact of glycolytic metabolism on prognosis has been poorly investigated in breast cancer. The aims of this study were threefold: to characterize the glycolytic phenotype of non-metastatic breast cancers, to identify new metabolic prognostic marker of survival in node negative breast cancers, and to analyze more precisely the most glycolytic tumor subgroup, the TNBC.

2. Methods

2.1. Patients, work-up and follow-up

Two independent cohorts (cohort A and B) of patients were analyzed. Patients were recorded retrospectively from a single-institution database from 1994 to 2002 for the cohort A, and from 2003 to 2009 for the cohort B (characteristics of population are described in Table 1). Inclusion criteria for cohort A were as follows: histological diagnosis of invasive breast carcinoma, available formalin-fixed paraffin-embedded tissues, female gender, node negative, no distant metastasis at diagnosis, no neoadjuvant treatment (either chemotherapy, hormonotherapy or radiotherapy). Inclusion criteria for the cohort B were the same as cohort A but also included all node positive patients.

Table 1
Patient Demographics and Clinical Characteristics for cohort A and B.

Demographic or clinical characteristic	Cohort A (n = 643)		Cohort B (n = 127)	
	No. of patients	(%)	No. of patients	(%)
Median age, years	62 (25–87)		63 (28–85)	
Median follow-up, months	124 (3–221)		54 (3–172)	
T stage				
pT1	499	77.6	71	55.9
pT2	139	21.6	50	39.4
pT3	1	0.2	1	0.9
pT4	2	0.3	4	2.9
Not available	2	0.3	1	0.9
N Stage				
N0	643	100	71	55.9
N1	0	0	37	29.1
N2	0	0	12	9.4
N3	0	0	6	4.7
Not available	0	0	1	0.9
SBR grade				
I	252	39.2	4	3.1
II	216	33.6	25	19.7
III	109	17	84	66.1
Not available	66	10.3	14	11.1
RH+	527	82	0	0
Her-2+	64	10	0	0
TNBC	52	8.1	127	100
Histology				
Carcinoma not otherwise specified	545	84.8	98	77.2
Lobular carcinoma	78	12.1	10	7.9
Others (medullary, colloid, metaplasic, cystic adenoid carcinoma, apocrin, sarcomatoid, metaplastic)	18	2.8	18	14
Not available	2	0.3	1	0.9

Cohort A and B encompassed 643 and 127 patients respectively. Population characteristics are depicted in Table 1. Adjuvant chemotherapy was given to patients with a bad prognosis (age <40 years, node positive, high grade, T3–T4, lymphovascular invasion). Radiotherapy was given when lumpectomy was performed or with mastectomy when there were also bad prognostic factors as mentioned previously (as well as multifocal disease). Of note, since inclusion of Her-2 patients occured before 2003, no patients received the adjuvant trastuzumab.

2.2. Immunohistochemistry

Immunohistochemical analysis was performed with the tissue-micro array (TMA) technique as described previously [16]. Three cores of 1 mm from different representative tumor areas were taken for each patient and observed by two pathologists (F.E., I.P.) concomitantly under the microscope.

Sections (3 μm thick) of formalin-fixed, paraffin-embedded tumor tissues were transferred to slides (X-Tra, Surgipath) and were air-dried overnight at 57 °C. Immunostaining was performed automatically with the Ventana device using the standard streptavidin–biotin complex method with 3,3′-diaminobenzidine chromogen. Hormone-sensitive (HR+) tumors were defined as tumors with expression of either nuclear estrogen or progesterone receptors (respectively ER and PR) in >1% of tumor nuclei. Her-2 positive tumors (Her-2+) were considered positive if scored as 3+, and fluorescent *in situ* hybridization with amplification ratio $\geqslant$2.0 was used in tumors scored as 2+. TNBC were considered as negative with the same criteria. The hormonal and Her-2 status was re-established with the same technique for all tumors.

2.3. Validation of antibodies against CAIX, MCT1, MCT4 and Basigin in xenograft tumors

We used antibodies against CAIX, MCT1, MCT4 and basigin (bsg) (Table 2) which have been previously shown to truly recognize these proteins of interest assessed by a positive signal when the given protein is expressed and loss of the signal when the protein is specifically knocked down (shRNA) or knocked out (ZFNs) [8,9,15].

2.4. Statistical analysis

Statistical comparisons were performed using Chi-Squared tests and log-rank tests for censored data. Statistical significance was achieved at $P < 0.05$. All statistical analyses were two-sided and performed using Statistical Package for the Social Sciences (SPSS), version 16.0. Local-free-relapse (LFR), metastasis-free survival (MFS), and overall survival (OS) were determined by Kaplan–Meier analysis. Correlation between variables was determined using the Spearman Rank test. Survival was calculated from the last day of treatment. Cox Regression was performed to assess independence of factors correlated in univariate analysis with survival. The following factors were screened as potential bad prognostic factor for survival: age <50 years, pT2-4 and pN+ stage, SBR grade III, Ki-67 >30% of nuclei (nuclear staining), CAIX positive (membranous staining in >5% of tumoural cells), MCT1 (membranous staining in >5% of tumoural cells), MCT4 (membranous staining in >5% tumoural cells), basigin (membranous staining in >5% of tumoural cells), LDHA (semi-quantitative scale: 2+ and 3+) and GLUT1 (membranous staining in >5% of tumoural cells).

Table 2
Source, dilution, and pretreatment of antibodies used for each marker.

Marker	Type	Reference	Buffer for retrieval antigen/duration of exposure	Dilution	Incubation duration
RE	Monoclonal	Ventana	CC1 30 min	No dilution	20 min
RP	Monoclonal	Ventana	CC1 30 min	No dilution	20 min
HER2/neu	Monoclonal	NCL-CB11 Novocastra Ventana	CC1 30 min	No dilution	20 min
CA IX	Monoclonal	MN75 Bayer	CC2 1 h	1/5000	32 min
GLUT1	Polyclonal	Ab15309 AbCam	CC1 1 h	1/750	32 min
MCT1	Polyclonal	Anti SLC 16A1 Sigma Prestige	CC2 1 h	1/100	1 h
MCT4	Polyclonal	Anti-SLC 16A3 Sigma Prestige	CC2 1 h	1/50	32 min
Basigin	Monoclonal	LS-C14277 Life span Biosciences	CC1 1 h	1/25	32 min
LDHA	Polyclonal	Ab47010 Abcam	CC1 30 min	1/300	1 h 30 min
Ki-67					

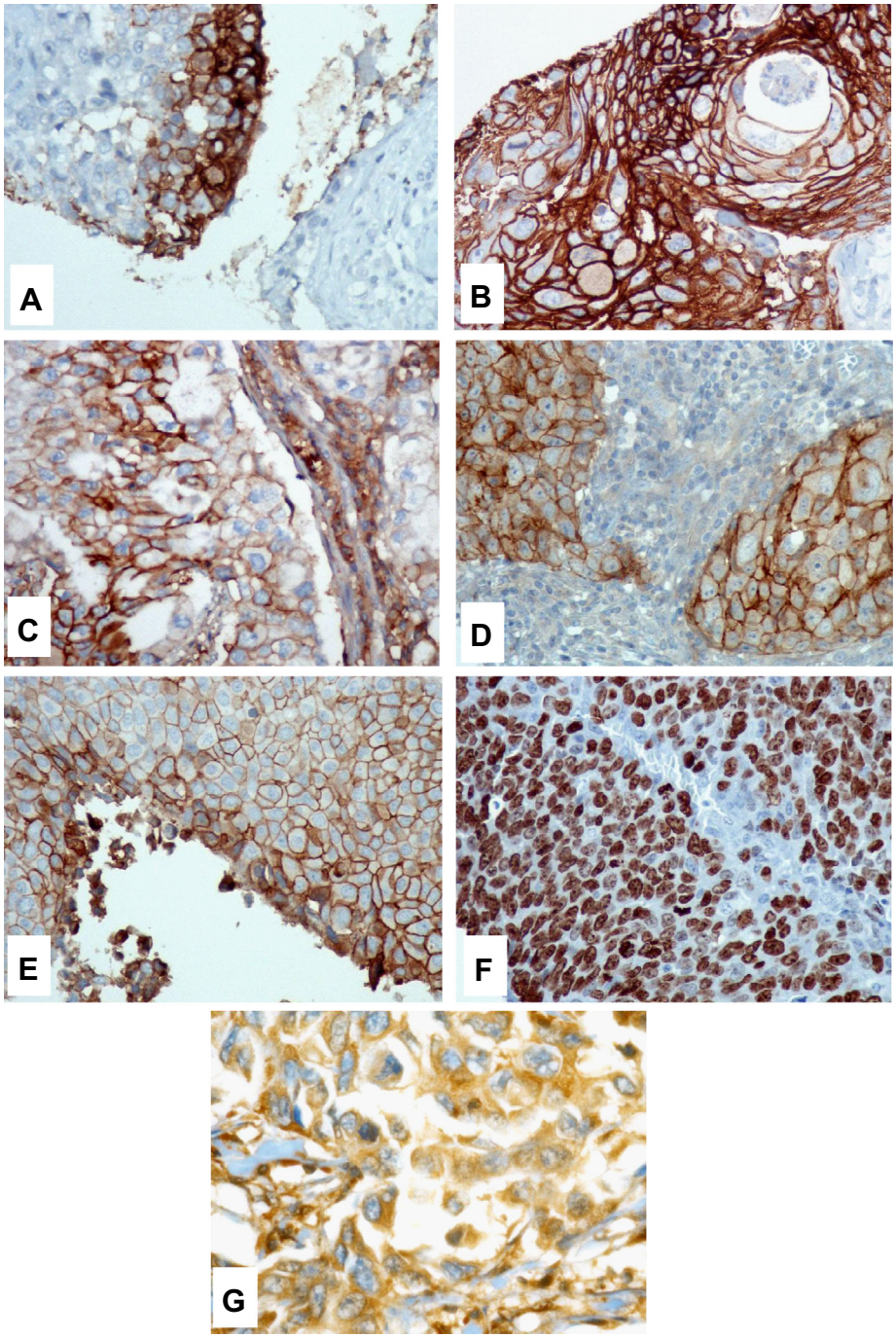

Fig. 1. Immunohistochemistry demonstrating staining patterns for glycolytic markers and Ki-67 (All images are at 400× magnification except for LDHA at 600×), (A) CAIX (membranous), (B) GLUT-1 (membranous), (C) Basigin (membranous), (D) MCT1 (membranous), (E) MCT4 (membranous), (F) Ki-67 (nuclear), (G) LDHA (cytoplasmic).

Table 3
Expression of glycolytic markers in cohort A and B.

Cohorts	CAIX	GLUT1	MCT1	MCT4	Basigin	LDHA 2+/3+	Ki-67 ⩾30%
Cohort A (N-breast cancer)							
RH+	14.1%	31.1%	40.9%	23.2%	40.7%	88.2%	6.7%
Her-2+	40.4%	66%	61.7%	36.7%	70.2%	94.1%	16.3%
TNBC	71.4%	86.4%	65.6%	58.1%	82.8%	87.1%	63.8%
P-value (chi-square)	<0.001	<0.001	<0.001	<0.001	<0.001	0.4	<0.001
Cohort B (TNBC)	64.6%	80.8%	61.4%	57.5%	80.6%	96.8%	45.7%

Table 4
Expression of glycolytic markers according to tumor characteristics.

Cohorts	pT1 vs pT2–4	Chi-square (*P*-value)	pN− vs pN+	Chi-square (*P*-value)	SBR I–II vs SBR III	Chi-square (*P*-value)	Ki67 <30% vs Ki67 ⩾30%	Chi-square (*P*-value)
Cohort A (N-breast cancer)								
CAIX	19.4%	0.002	NA	NA	15.1%	<0.001	17.4%	<0.001
	31.4%				54.1%		59.2%	
GLUT-1	36.8%	0.009			31.7%	<0.001	35.4%	<0.001
	49.6%				77.6%		76.4%	
MCT1	43.1%	0.08			40.7%	<0.001	39.5%	<0.001
	51.5%				65%		82.4%	
MCT4	25.4%	0.01			20.7%	<0.001	23.9%	<0.001
	35.5%				60.2%		61.8%	
Basigin	46.4%	0.4			40.3%	<0.001	42.7%	<0.001
	50%				83.3%		86.1%	
LDHA 2+/3+	89.1%	0.3			90.4%	0.6	90.2%	0.6
	86.4%				88.9%		92%	
Cohort B (TNBC)								
CAIX	66.2%	0.7	66.2%	0.7	65.5%	0.9	60.7%	0.3
	63.6%		63.6%		66.7%		69%	
GLUT-1	74.6%	0.02	80%	0.6	51.7%	<0.001	71.7%	0.003
	90.6%		83.3%		92.8%		93%	
MCT1	53.5%	0.01	64.8%	0.4	31%	<0.001	44.3%	<0.001
	72.7%		58.2%		72.6%		82.8%	
MCT4	55.7%	0.4	56.3%	0.5	32.1%	0.001	46.7%	0.01
	61.8%		61.1%		66.7%		69%	
Basigin	72.9%	0.006	81.2%	0.9	50%	<0.001	72.9%	0.004
	92.5%		81.5%		90.4%		93%	
LDHA 2+/3+	95.7%	0.4	97.2%	0.7	93.1%	0.2	95.1%	0.09
	98.2%		96.3%		97.6%		100%	

NA = not applicable

3. Results

3.1. Expression of glycolytic markers

All glycolytic markers analyzed, except for LDHA located in the cytosol, are proteins located at the plasma membrane as shown in the immunostaining depicted in Fig. 1. Expression patterns of glycolytic markers are described in Table 3. Except for LDHA expression, we observed a strongly significant difference between the 3 breast cancer subgroups with a progressive increase of the expression of glycolytic markers from RH+ to Her-2+ and finally TNBC subgroups. Expression of the glycolytic markers was approximately 14–40%, 40–70% and 58–86% in RH+, Her-2+ and TNBC populations respectively. LDH-A was ubiquitous and expressed at a very high rate in all breast tumors. Expression of membrane-bound glycolytic markers was very similar between the TNBC subgroup of cohort A and the TNBC cohort B as shown in Table 3. Node involvement was not associated with different glycolytic features (Table 4, $P > 0.3$).

Table 4 shows the analysis of glycolytic markers with respect to tumor characteristics. Glycolytic markers were strongly expressed in large, high grade, and highly proliferative tumors in both cohorts. As mentioned previously, no glycolytic markers were associated with node involvement (cohort B).

Basigin, MCT1 and MCT4 are functionally related as basigin (also known as CD147 and EMMPRIN) is the main accessory glycoprotein required for proper folding of MCTs in the endoplasmic reticulum and trafficking to the plasma [5,9]. Tumors that expressed MCT1 also expressed basigin in 78.3% of the cohort A (55/254 MCT1+ did not) and 97.4% of the cohort B (2/77 MCT1+ did not). Tumors that expressed MCT4 also expressed basigin in 73.9% of the cohort A (43/165 MCT4+ did not) and 90.3% of the cohort B (7/72 MCT4+ did not). Among the TNBC subgroups of cohort A and B respectively 45.2% and 40.9% patients expressed both MCT1 and MCT4 while in the whole cohort A only 15.6% expressed both MCT1 and MCT4.

GLUT1 facilitates glucose uptake, which is typically increased for tumor cells that have switched to fermentative glycolysis and production of lactic acid (Warburg effect). Among the TNBC subgroup of cohort A, 97.4% and 91.4% of the tumors expressed GLUT1 when expressing respectively MCT1 or MCT4. These results were similar in cohort B: 94.7% and 91.5% of TNBC tumors expressed GLUT1 when expressing respectively MCT1 or MCT4.

Stromal staining was often observed for MCT4 either in lymphocytes or fibroblasts (Fig. 2). There were 4 different groups of MCT4 staining: tumoural (Tu), stromal (St), tumoural and stromal (Tu/St), or none (Table 3, Fig. 2).

3.2. Survival and prognostic factors for MFS and OS

After a median follow-up of 124 (3–221) months in cohort A and 54 months (3–172) in cohort B, we observed 43 and 23 local relapses, 60 and 38 metastatic relapses, 50 and 29 deaths due to cancer in cohorts A and B respectively. This translated into a 5-year

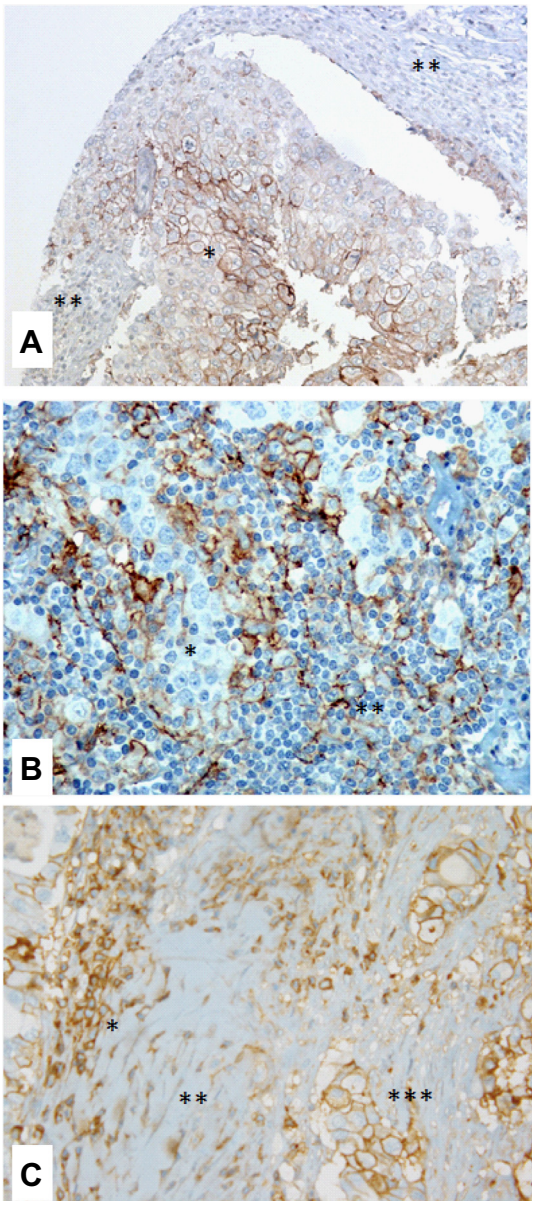

Fig. 2. MCT4 Immunohistochemistry in 3 different tumours: tumoural positive/stromal negative for MCT4 (A, * = tumoural cells, ** = stromal cells), tumoural negative/stromal positive for MCT4 (B, * = tumoural cells, ** = stromal cells); tumoural and stromal positive (C, * = lymphocytes, ** = fibroblasts, *** = tumoural cells).

rate for LFR, MFS and OS of respectively 97.3% (cohort A) and 81.4% (cohort B), 95.3% (cohort A) and 67.7% (cohort B), 95.2% (cohort A) and 76.5% (cohort B).

In univariate analysis the following factors were considered as prognostic factors for MFS in cohort A: pT stage, SBR grade, tumoural MCT4 staining (Fig. 3C; when comparing over the 4 strata MCT4 positivity was not prognostic, $P = 0.08$, Fig. 3A), Ki67 and being RH+, Her-2+ or TNBC. In multivariate analysis with stepwise Cox regression (forward and backward, wald) SBR grade and tumoural MCT4 staining were identified as independent prognostic factors for MFS (Table 5).

In univariate analysis the following factors were considered as prognostic factors for MFS in cohort B: pT stage, pN stage, tumoral MCT4 staining (Fig. 3D; when comparing over the 4 strata MCT4 positivity was not prognostic, $P = 0.058$, Fig. 3B), GLUT1 positivity and basigin positivity. pN, pT and tumoral MCT4 staining were considered as independent prognostic factors for MFS in cohort B (Table 5).

In univariate analysis the following factors were considered as prognostic factors for OS in cohort A: age, pT stage, SBR grade, tumoral MCT4 staining (when comparing over the 4 strata MCT4 positivity was not prognostic, $P = 0.07$), Ki67 and being RH+, Her-2+ or TNBC but only SBR grade and tumoural MCT4 staining were considered as independent prognostic factor in multivariate analysis (Table 6).

In univariate analysis pT stage, pN stage and tumoral MCT4 (when comparing over the 4 strata MCT4 positivity was not prognostic, $P = 0.2$) were found to be associated with OS in cohort B and all retained independent prognostic value in multivariate analysis (Table 6).

Of note the Ki-67 prognostic impact was tested in cohort B with several cut-off points (20%, 30%, 40%, 50% and 60%) but none reached statistical significance ($P > 0.5$).

4. Discussion

Glycolytic metabolism is used in daily clinical practice for diagnosis with ^{18}FDG PET-scanning. This technique exploits the avidity of tumoural cells for glucose allowing more accurate diagnosis by detection of some metastases or primary tumors that could not be detected by CT-scan. Tumor cells typically consume much more glucose than normal cells and this characteristic is used for diagnosis but not for treatment or prognosis up to now. This "glucose addiction" also leads to differential expression of a large number of proteins specialized for acid detoxification in order to maintain an alkaline tumoural intracellular pH (pH_i) compared to the very acidic extracellular pH [6]. Previous studies utilizing ^{18}FDG PET scan in early breast cancer showed ^{18}FDG uptake in almost all breast tumors but with a very different SUV according to breast subgroup. TNBC had the highest baseline SUV values (9.8) followed by Her-2 positive tumors (6.6) and RH+ tumors (6.3) ($P = 0.001$) in the study of Keam et al. [13]. In this study, highly proliferative tumors also harbored a higher SUV value (8.5 vs 6.2, $P = 0.01$). Another study showed higher SUV values in tumors with higher SBR grade (SBR I: 2.7, SBR II: 3.1, SBR III: 9.1, $P = 0.01$) and with larger size (cut-off 2 cm: 5.4 vs 9.2 in TNBC, and 1.9 vs 3.5 in non-TNBC, $P < 0.05$) [12]. Our study is the first exhaustive analysis of the expression of glycolytic markers in breast tumors in a large data set. Our results confirmed what was observed with ^{18}FDG PET-scan but with a stronger differential expression between RH+ being sometimes glycolytic, Her-2 being often glycolytic and TNBC being almost systematically glycolytic (expression of glycolytic markers of respectively 14–40%, 40–70% and 58–86%, Table 3). Tumors with large size, higher grade, and a high Ki-67 also systematically expressed more frequently all glycolytic markers (Table 4). This was confirmed in an independent cohort of TNBC for all markers but not for Ki-67, which is perhaps due to the majority of TNBC being highly proliferative which may reduce the statistical power. In cohort B, none of the markers were associated with node involvement, which seems to occur independently from the glycolytic phenotype of the primary tumor.

LDH-A was ubiquitous and expressed at a very high rate (around 90–95% at a moderate/high intensity) in all breast tumors. It was consequently not associated with tumor characteristics. All breast tumor cells therefore appear to have the enzymatic equipment to produce ATP through fermentative glycolysis.

Regarding survival some glycolytic markers correlated with MFS or OS. In cohort A tumoural MCT4 staining correlated independently with MFS (Table 5) and OS (Table 6) while tumoural MCT4 staining, GLUT1 and basigin positivity correlated in univariate analysis for MFS in cohort B, but not in multivariate analysis except for tumoural MCT4.

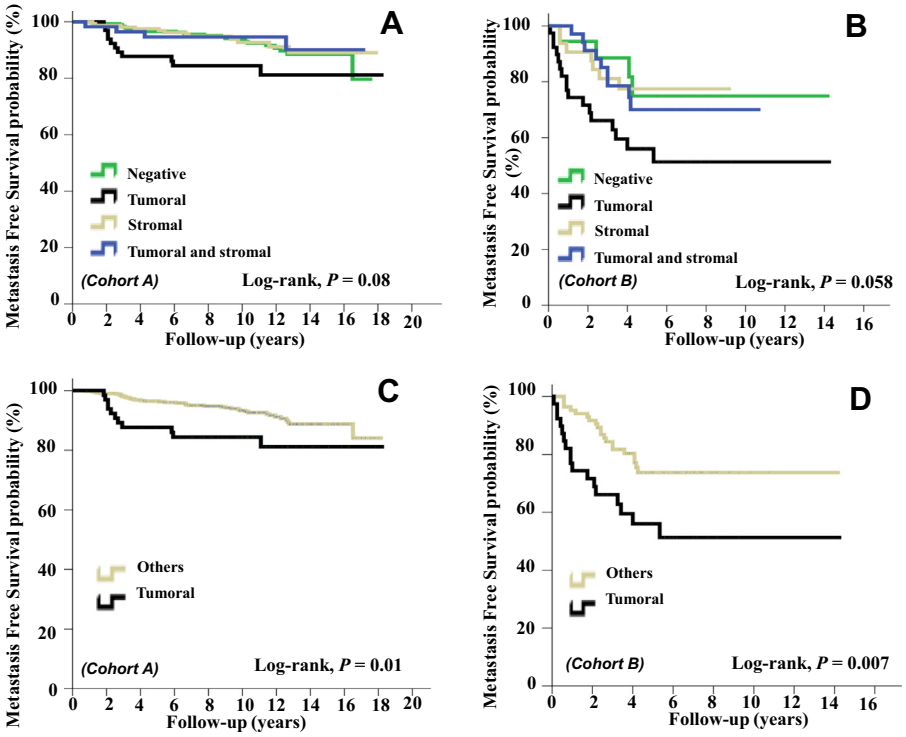

Fig. 3. Prognosis impact for metastasis-free survival following MCT4 staining patterns in tumors, stroma and both in cohort A (A and C) and cohort B (B and D).

Table 5
Prognostic factors for Metastasis-Free-Survival in cohort A and B.

Variable	5-year MFS	Log-rank	Cox regression	Stepwise Cox regression (forward and backward, wald)
Cohort A (N- breast cancer)				
pT1	96.8%	$P = 0.002$	$P = 0.2$	
pT2–T4	89.9%			
SBR I–II	97.2%	$P < 0.001$	0.4	0.38
SBR III	93.4%		[0.19–0.83]	[0.18–0.77]
			$P = 0.01$	$P = 0.007$
MCT4 St – Tu/St – Negative	96.5%	$P = 0.01$	$P = 0.09$	0.47
MCT4 Tu	87.7%			[0.24–0.92]
				$P = 0.02$
Ki-67 < 30%	95.9%	$P = 0.02$	$P = 0.1$	
Ki-67 ⩾ 30%	89.3%			
RH+	96.3%	$P = 0.007$	$P = 0.4$	
TNBC	92%			
Her-2+	88.5%			
Cohort B (TNBC)				
pT1	79.6%	$P = 0.001$	0.37	0.34
pT2–T4	50.8%		[0.18–0.75]	[0.17–0.69]
			$P = 0.006$	$P = 0.003$
pN0	75%	$P = 0.01$	0.45	0.43
pN1-3	57.1%		[0.23–0.89]	[0.22–0.87]
			$P = 0.02$	$P = 0.01$
GLUT1 negative	85.6%	$P = 0.03$	$P = 0.2$	
GLUT1 positive	63.8%			
MCT4 St – Tu/St – Negative	73.7%	$P = 0.007$	0.46	0.46
MCT4 Tu	56%		[0.23–0.91]	[0.24–0.91]
			$P = 0.02$	$P = 0.02$
Basigin negative	86.4%	$P = 0.05$	$P = 0.8$	
Basigin positive	62.6%			

The most important finding of this study is that tumoural MCT4 staining emerged as a new independent prognostic factor for survival in node negative breast cancer, as well as in TNBC. MCT4 is a specialized protein for lactate export. The affinity of MCT4 for lactate transport is lower than MCT1 (*Km* of 28 mM for MCT4 vs 4.5 mM for MCT1 [5]) but MCT1 also transports other

Table 6
Prognostic factors for Overall Survival in cohort A and B.

Variable	5-year MFS	Log-rank	Cox regression	Stepwise Cox regression (forward and backward, wald)
Cohort A (N-breast cancer)				
Age < 50 years	98.3%	$P < 0.001$	$P = 0.2$	
Age ⩾ 50 years	94.1%			
pT1	96.4%	$P < 0.001$	$P = 0.1$	
pT2–4	89.4%			
SBR I–II	97.2%	$P < 0.001$	0.23	0.22
SBR III	93.5%		[0.09–0.61]	[0.09–0.57]
			$P = 0.003$	$P = 0.002$
MCT4 St – Tu/St – Negative	96.5%	$P = 0.01$	0.42	0.38
MCT4 Tu	87.9%		[0.2–0.88]	[0.19–0.77]
			$P = 0.02$	$P = 0.007$
Ki-67 < 30%	95.5%	$P = 0.01$	$P = 0.6$	
Ki-67 ⩾ 30%	89.5%			
RH+	96.2%	$P < 0.001$	$P = 0.3$	
TNBC	87.5%			
Her-2+	90.4%			
Cohort B (TNBC)				
pT1	86.3%	$P = 0.003$	0.36	0.36
pT2–T4	63%		[0.17–0.76]	[0.17–0.76]
			$P = 0.008$	$P = 0.008$
pN0	87%	$P = 0.01$	0.47	0.47
pN1-3	62.9%		[0.23–0.99]	[0.23–0.99]
			$P = 0.04$	$P = 0.04$
GLUT1 negative	86.8%	$P = 0.13$	Not included	
GLUT1 positive	74%			
MCT4 St – Tu/St – Negative	80.2%	$P = 0.04$	0.49	0.49
MCT4 Tu	70.3%		[0.24–0.99]	[0.24–0.99]
			$P = 0.04$	$P = 0.04$
Basigin negative	82.6%	$P = 0.18$	Not included	
Basigin positive	75%			

monocarboxylates such as pyruvate. Exporting pyruvate is inefficient for the bioenergetics of glycolysis that relies on pyruvate reduction into lactate to renew NAD+. Indeed most glycolytic cells grow slightly faster when treated with MCT1 inhibitor *in vitro* (our unpublished results). MCT transport is bi-directional and allows import and export of pyruvate and lactate as oxidative fuels *via* high affinity-transporters such as MCT1, 2 in several cells including neurons and muscle cells, and in the metabolic reprogramming through the lactate shuttle within tumor–stroma interplay [7,17,18]. The ability of MCT1 to export pyruvate might explain the weak prognostic impact of MCT1 staining in the present study. Among the glycolytic proteins tested in this study, MCT4 is one of the main proteins that strongly relies on HIF-1 stabilization for its induction [5]. There are several explanations to understand why tumoural MCT4 staining is correlated with a more aggressive tumor phenotype. Firstly, MCT4 expression was shown to induce an alkaline pH_i as a result of accelerate lactic acid export leading to net increase in glycolytic rate and tumor growth [19]. Secondly, tumoural MCT4 expression may reflect strong activation of HIF-1, which activates the epithelial to mesenchymal transition [14] facilitating tumor aggressiveness and metastasis. Thirdly, MCT4 staining could reflect enrichment in putative tumoural stem cells. Indeed in glioblastoma cell lines it was recently demonstrated that silencing of MCT4 could reduce CD133 expression, a well-known stem cell marker [20]. This translated into reduce *in vitro* and *in vivo* tumor growth. Finally, a more mechanistic explanation for the prognostic impact of tumoural MCT4 staining, through active glycolysis and lactic acid extrusion, could be due to the decreased ability of chemotherapeutic drugs to be active in a more acidic microenvironment and alkaline intracellular milieu [21].

Interestingly, tumors that expressed stromal MCT4 staining did not harbor worse outcome in the present study, regardless of the tumoural status. Stromal staining could happen either in lymphocytes or fibroblasts meaning that better outcome could be due to MCT4 expression in one or both of these cell types. Numerous studies reported previously that tumor infiltrated lymphocytes (TIL) are indicators of a much better outcome especially in TNBC [22] or estrogen receptor negative breast cancers [23]. It is also well known that lymphocytes are highly glycolytic cells with strong MCT4 expression [5]. Surprisingly another study showed that tumoural expression of MCT4 correlated with better survival while stromal MCT4 expression correlated with worse prognosis [17]. In this study however, the authors did not analyze tumoural and stromal expression together, which may explain these opposing results. In this study, prognosis value of MCT4 expression was also not adjusted for grade, size or Ki-67 in multivariate analysis.

CAIX expression did not correlate with survival in our study. Membrane carbonic anhydrases allows extracellular hydration of CO_2 into bicarbonate and H^+ increasing by this way passive extrusion of CO_2 from intracellular to extracellular space whereas reuptake of bicarbonate (weak base) by bicarbonate transporters will contribute to maintenance of an alkaline pH_i within an acidic microenvironment [6,24]. In a previous study of 132 invasive breast carcinomas, it was showed that overexpression of HIF-1α and CA IX correlated with a poor prognosis. However only HIF-1α was an independent prognostic factor for distant metastasis-free survival and disease-free survival in multivariate analysis [15]. The absence of correlation of CAIX staining with survival was latter confirmed in a cohort of 120 patients with TNBC breast cancer [25] and could reflect the strong anaerobic/glycolytic

phenotype of TNBC tumors which seems to rely more on lactic acid production (and lactic acid export) than on OXPHOS.

Another surprising finding in our study was the absence of prognostic value for Ki-67 staining in the TNBC cohort. This was already reported in another study, which also showed absence of correlation between survival and mitotic score in a cohort of 244 TNBC breast cancers [26]. This could be due to the very high rate of proliferating tumors in this subgroup that decreases the statistical power of the analysis.

In conclusion this study provides a new independent prognostic factor for survival in node negative breast cancer and TNBC breast cancer especially. This prognostic factor may be useful to indicate the requirement of adjuvant chemotherapy in those patients with tumoural/non stromal MCT4 staining. This finding however requires future validation in pathologic blocks derived from randomized trials. Direct targeting of MCT4 also seems to be a promising anti-cancer strategy. A previous study used non-specific inhibitors of MCTs (alpha-cyano-4-hydroxycinnamate (CHC), Quercetin and Lonidamine) and showed potential antiproliferation activity of this strategy *in vitro* [27]. However, two other studies using a specific inhibitor of MCT1 in purely glycolytic cells lacking MCT4 showed a rapid decrease in intracellular pH and glycolysis leading to tumor growth arrest [9,10].

Glycolytic metabolism was proposed as a new hallmark of cancer by Hanahan and Weinberg [28] but up to now there are no clinical applications and specific inhibitors targeting glycolytic major components such as MCT4 are not only warranted but in the process of active development in several pharmacological companies.

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References

[1] G. Kroemer, J. Pouyssegur, Tumor cell metabolism: cancer's Achilles' heel, Cancer Cell 13 (2008) 472–482.
[2] M.G. Vander Heiden, L.C. Cantley, C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation, Science 324 (2009) 1029–1033.
[3] O. Warburg, On respiratory impairment in cancer cells, Science 124 (1956) 269–270.
[4] C.T. Supuran, Carbonic anhydrases: novel therapeutic applications for inhibitors and activators, Nat. Rev. Drug Discov. 7 (2008) 168–181.
[5] A.P. Halestrap, The monocarboxylate transporter family – structure and functional characterization, IUBMB Life 64 (2012) 1–9.
[6] S.K. Parks, J. Chiche, J. Pouyssegur, Disrupting proton dynamics and energy metabolism for cancer therapy, Nat. Rev. Cancer 13 (2013) 611–623.
[7] P. Sonveaux, F. Vegran, T. Schroeder, M.C. Wergin, J. Verrax, Z.N. Rabbani, C.J. De Saedeleer, K.M. Kennedy, C. Diepart, B.F. Jordan, M.J. Kelley, B. Gallez, M.L. Wahl, O. Feron, M.W. Dewhirst, Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice, J. Clin. Invest. 118 (2008) 3930–3942.
[8] J. Chiche, K. Ilc, J. Laferriere, E. Trottier, F. Dayan, N.M. Mazure, M.C. Brahimi-Horn, J. Pouyssegur, Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH, Cancer Res. 69 (2009) 358–368.
[9] R. Le Floch, J. Chiche, I. Marchiq, T. Naiken, K. Ilc, C.M. Murray, S.E. Critchlow, D. Roux, M.P. Simon, J. Pouyssegur, CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 16663–16668.
[10] J.R. Doherty, C. Yang, K.E. Scott, M.D. Cameron, M. Fallahi, W. Li, M.A. Hall, A.L. Amelio, J.K. Mishra, F. Li, M. Tortosa, H.M. Genau, R.J. Rounbehler, Y. Lu, C.V. Dang, K.G. Kumar, A.A. Butler, T.D. Bannister, A.T. Hooper, K. UnsalKacmaz, W.R. Roush, J.L. Cleveland, Blocking lactate export by inhibiting the Myc target MCT1 disables glycolysis and glutathione synthesis, Cancer Res. (2013).
[11] A. Schulze, A.L. Harris, How cancer metabolism is tuned for proliferation and vulnerable to disruption, Nature 491 (2012) 364–373.
[12] S. Basu, W. Chen, J. Tchou, A. Mavi, T. Cermik, B. Czerniecki, M. Schnall, A. Alavi, Comparison of triple-negative and estrogen receptor-positive/progesterone receptor-positive/HER2-negative breast carcinoma using quantitative fluorine-18 fluorodeoxyglucose/positron emission tomography imaging parameters: a potentially useful method for disease characterization, Cancer 112 (2008) 995–1000.
[13] B. Keam, S.A. Im, Y. Koh, S.W. Han, D.Y. Oh, N. Cho, J.H. Kim, W. Han, K.W. Kang, W.K. Moon, T.Y. Kim, I.A. Park, D.Y. Noh, J.K. Chung, Y.J. Bang, Early metabolic response using FDG PET/CT and molecular phenotypes of breast cancer treated with neoadjuvant chemotherapy, BMC Cancer 11 (2011) 452.
[14] J. Pouyssegur, F. Dayan, N.M. Mazure, Hypoxia signalling in cancer and approaches to enforce tumour regression, Nature 441 (2006) 437–443.
[15] C. Trastour, E. Benizri, F. Ettore, A. Ramaioli, E. Chamorey, J. Pouyssegur, E. Berra, HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome, Int. J. Cancer 120 (2007) 1451–1458.
[16] M.C. Cheang, D.O. Treaba, C.H. Speers, I.A. Olivotto, C.D. Bajdik, S.K. Chia, L.C. Goldstein, K.A. Gelmon, D. Huntsman, C.B. Gilks, T.O. Nielsen, A.M. Gown, Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival, J. Clin. Oncol. 24 (2006) 5637–5644.
[17] A.K. Witkiewicz, D. Whitaker-Menezes, A. Dasgupta, N.J. Philp, Z. Lin, R. Gandara, S. Sneddon, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti, Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers, Cell Cycle 11 (2012) 1108–1117.
[18] T. Fiaschi, A. Marini, E. Giannoni, M.L. Taddei, P. Gandellini, A. De Donatis, M. Lanciotti, S. Serni, P. Cirri, P. Chiarugi, Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay, Cancer Res. 72 (2012) 5130–5140.
[19] J. Chiche, Y. Le Fur, C. Vilmen, F. Frassineti, L. Daniel, A.P. Halestrap, P.J. Cozzone, J. Pouyssegur, N.W. Lutz, In vivo pH in metabolic-defective Ras-transformed fibroblast tumors: key role of the monocarboxylate transporter, MCT4, for inducing an alkaline intracellular pH, Int. J. Cancer 130 (2012) 1511–1520.
[20] K.S. Lim, K.J. Lim, A.C. Price, B.A. Orr, C.G. Eberhart, E.E. Bar, Inhibition of monocarboxylate transporter-4 depletes stem-like glioblastoma cells and inhibits HIF transcriptional response in a lactate-independent manner, Oncogene (2013).
[21] J.K. Saggar, M. Yu, Q. Tan, I.F. Tannock, The tumor microenvironment and strategies to improve drug distribution, Front. Oncol. 3 (2013) 154.
[22] S. Loi, N. Sirtaine, F. Piette, R. Salgado, G. Viale, F. Van Eenoo, G. Rouas, P. Francis, J.P. Crown, E. Hitre, E. de Azambuja, E. Quinaux, A. Di Leo, S. Michiels, M.J. Piccart, C. Sotiriou, Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02–98, J. Clin. Oncol. 31 (2013) 860–867.
[23] N.R. West, S.E. Kost, S.D. Martin, K. Milne, R.J. Deleeuw, B.H. Nelson, P.H. Watson, Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer, Br. J. Cancer 108 (2013) 155–162.
[24] P. Swietach, A. Hulikova, R.D. Vaughan-Jones, A.L. Harris, New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation, Oncogene 29 (2010) 6509–6521.
[25] V.M. Neumeister, C.A. Sullivan, R. Lindner, K. Lezon-Geyda, J. Li, J. Zavada, M. Martel, P.M. Glazer, D.P. Tuck, D.L. Rimm, L. Harris, Hypoxia-induced protein CAIX is associated with somatic loss of BRCA1 protein and pathway activity in triple negative breast cancer, Breast Cancer Res. Treat. 136 (2012) 67–75.
[26] M.A. Aleskandarany, A.R. Green, A.A. Benhasouna, F.F. Barros, K. Neal, J.S. Reis-Filho, I.O. Ellis, E.A. Rakha, Prognostic value of proliferation assay in the luminal, HER2-positive, and triple-negative biologic classes of breast cancer, Breast Cancer Res. 14 (2012) R3.
[27] F. Morais-Santos, V. Miranda-Goncalves, S. Pinheiro, A.F. Vieira, J. Paredes, F. Schmitt, F. Baltazar, C. Pinheiro, Differential sensitivities to lactate transport inhibitors of breast cancer cell lines, Endocr. Relat. Cancer (2013).
[28] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.