Chapter 10

HIF-1α and PKM2 are important drivers of age associated clinical functional decline and disease in the elderly breast cancer population: A FOCUS study analysis

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ABSTRACT

Introduction

HIF-1α is over-expressed in the majority of tumors. Evidence exists for HIF-1α accumulation during aging, a process that is also associated with higher cancer risk. In this study, we investigated the difference in expression of HIF-1α and its associated target genes in both normal and cancer tissue from middle-aged and old breast cancer patients. The aim of this study was to determine whether the level of expression is associated with patient characteristics associated with aging and outcome.

Material and Methods

120 patients, aged ≥ 65 years, with invasive, non-metastatic breast cancer with formalin fixed paraffin embedded tumor and normal breast samples available were included. On both tumor and normal tissue, total RNA was extracted and RT-PCR was performed for determination of HIF-1α and its associated target genes. Immunohistochemical stainings for HIF1-α and PKM2 were performed on both tumor and normal tissue. Based on the mean value, patients were stratified into two age groups: 65 to 80 years and ≥ 80 years. The difference in mRNA expression per primer between middle-aged and old patients per tissue type and associations with clinicopathological parameters were evaluated. Clinical endpoints examined were Overall Survival, Disease Free Survival, and Relapse Free Period.

Results

Higher mRNA expression of HIF1-α (p=0.017), GAPDH (p=0.003), PKM2 (p=0.069) and VEGFA (p=0.071 was seen in normal breast tissue of the older patients compared to the middle-aged. Upregulation of HIF1-α targets in normal breast tissue was significantly associated with different patient characteristics associated with clinical deterioration. Compared to normal breast tissue, tumor tissue of middle-aged patients showed a significant increase of HIF1-α (p=0.0011), GAPDH (p=0.0260) and TFAM (p=0.0171). This significant increase in the tumor tissue was also seen in patients older than 80 years for HIF1-α (p=0.0242) and TFAM (p=0.0041). High HIF1-α (HR1.65, 95%CI: 0.77-12.08, p=0.06) and PKM2 (HR1.69, 95CI: 0.95-3.03, p=0.08) mRNA expression in normal breast tissue showed a statistical trend for overall survival. High PKM2 (HR1.72, 95%CI:0.92-3.22, p=0.087) and VEGFA (HR2.07, 95%CI:1.01-4.14, p=0.039) mRNA expression in the breast tumor were associated with overall survival in univariate analyses, but lost their significance in the adjusted analyses.

Conclusion

This study supports the hypothesis that reversing or halting metabolic changes during aging possess the potential to benefit individuals as they reach an age where the chance of tumorigenesis increases exponentially. More research is needed to elucidate the potential contribution of age-related changes in HIF1-α and PKM2.

INTRODUCTION

Of all the factors that contribute to cancer, aging is the most potent but the reasons are still debated [1]. The most prominent explanation is the so-called multi-hit or Knudson hypothesis, which states that cancer occurs more frequently as we age because time is necessary for genetic mutations to accumulate and exceed a mutagenic threshold [2]. This hypothesis however fails to adequately explain why cancer risk is greatly reduced by calorie restriction and physical exercise [3]. Wu *et al.* proposed that a decline in metabolic homeostasis with age is a major contributor to increased cancer rate during aging [4]. In support of this hypothesis is the strong association between cancer and type 2 diabetes, obesity, and molecules that modulate energy utilization, such as metformin and resveratrol [3;5-9].

Under normal conditions healthy cells metabolize glucose by oxidative phosphorylation for efficient energy production whereas tumor cells preferentially metabolize glucose by aerobic glycolysis, also known as the Warburg effect. This produces less energy but facilitates rapid proliferation by enabling cells to incorporate metabolites from glycolysis [10;11].

Recently, evidence has emerged that the age related decline of metabolic homeostasis in healthy tissue is a driver of tumorigenesis. Gomes *et al.*, showed that in old mice a pseudohypoxic state causes Warburg-like metabolic reprogramming in normal tissue, resulting in disruption of mitochondrial homeostasis [12], a hallmark of aging [13]. Normally, in the absence of oxygen, the hypoxia-inducible factor HIF-1 binds to hypoxia-response elements (HREs), and activates the expression of numerous hypoxia-response genes [14]. Gomes *et al.* have suggested that this age-related decline in metabolic homeostasis induces a carcinogenic environment, partly due to an increase of reactive oxygen species (ROS), well known for its mutagenic potential, and thus might be an important reason for the high cancer incidence seen in the older population [4;12].

The metabolic shift away from oxidative phosphorylation to aerobic glycolysis is partly achieved and dependent on the glycolytic enzyme pyruvate kinase (PK) [10]. The existence of different PK isoforms (L, R, M1 and M2) reflects the importance of the last step of glycolysis to cope with the differential metabolic requirements of the cells [15]. The PKM1/M2 isoforms are generated through alternative splicing of two mutually exclusive exons by heterogenous nuclear ribonuclearprotein (hnRNP (hnRNPA1 and hnRNPA2)) and polypyrimidine tract binding protein (PTB) [10]. Normal cells express the pyruvate kinase M1 isoform (PKM1). As tumor cells shift away from oxidative phosphorylation (OXPHOS) toward anaerobic glycolysis, they predominantly express the M2 isoform (PKM2). The latter catalyzes the last step of glycolysis and reprograms the glycolytic flux to feed the special metabolic demands of proliferating cells [10]. It is for this reason that, over the last

decades, PKM2 has identified itself as a promising therapeutic target for cancer treatment, but could also potentially contribute to anti-aging interventions.

In this current study, we investigated the difference in expression of HIF-1α and its associated target genes, including PKM1 and 2, in both normal and cancer tissue from young and old breast cancer patients to determine whether their level of expression is associated with clinical characteristics associated with aging and outcome.

MATERIAL AND METHODS

Patients and tumors

For this study, 120 patients with invasive, non-metastatic breast cancer from the FOCUS cohort (Female breast cancer in the elderly, Optimizing Clinical guidelines USing clinic-pathological and molecular data) who received surgery and had formalin fixed paraffin embedded (FFPE) intra-operative tumor and normal breast samples available with successful determination of HIF-1α and its associated target genes were included. The FOCUS cohort has been described extensively in previous publications [16]. In brief, the cohort consists of all post-menopausal women, aged ≥ 65 years at time of diagnosis, with invasive and *in situ* breast cancer, diagnosed and treated between 1997 and 2004 in the South Western region of The Netherlands. Follow-up on survival status was available until the 1st of January 2013. All tissue samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Gene expression and mtDNA analysis

Total RNA from FFPE normal breast tissue and breast tumor tissue was extracted using the RNeasy FFPE kit (QIAGEN) according to the supplier's instructions. RNA samples were quantified using the Nanodrop 1000spectrophotometer (Thermo Scientific). cDNA was synthesized with the iSCRIPT cDNA synthesis kit (BioRad) using 200ng of RNA. Quantitative Real Time-PCR (RT-PCR) reactions were performed using 1uM of primers and LightCycler® 480 SYBR Green Master (Roche) on a lightcycler® 480 detection system (Roche). Calculations were performed by a comparative method ($2^{-\Delta CT}$) using Tubulin as an internal control. Primers for HIF-1α and its associated metabolic target genes, and mitochondrially and nuclear encoded OXPHOS genes were designed using the IDT software [14;17]. Primer sequences can be found in Table 1.

Immunohistochemistry for HIF1-α and PKM2

The immunohistochemical staining against HIF1-α was performed on tissue sections of intra-operatively derived FFPE tumor material and normal tissue of the FOCUS cohort.

Table 1: primer sequences used for RT-PCR

	Forward	Reverse
ATP-6	ACACCCCTTATCCCCATACTAG	AGTAATGTTAGCGGTTAGGCG
COX-1	CTTCGTCTGATCCGTCCTAATC	TTGAGGTTGCGGTCTGTTAG
CYTB	CCATACATTGGGACAGACCTAG	AGGGCAAGATGAAGTGAAAGG
ND1	CCTCTCCACCCTTATCACAAC	GTTGGTCTCTGCTAGTGTGG
GAPDH	AGAACGGGAAGCTTGTCATC	CATCGCCCCACTTGATTTTG
HIF1α	CCGCTGGAGACACAATCATATC	ACTTCCTCAAGTTGCTGGTC
HK-2	GAATTTGATGTGGCTGTGGATG	GTTACGGACAATCTCACCCAG
HMOX-1	TCAGGCAGAGGGTGATAGAAG	TTGGTGTCATGGGTCAGC
LDHA	AGATAAGGAACAGTGGAAAGAGG	CCAATAGCCCAGGATGTGTAG
PKM-1	ACCGCAAGCTGTTTGAAGAA	TCCATGAGGTCTGTGGAGTG
PKM-2	GAGGCCTCCTTCAAGTGCT	CCAGACTTGGTGAGGACGAT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
TFAM	CTACAGAACTAATTAGAAGAATTGCCC	CTCCGCCCTATAAGCATCTTG
COX-4	CCATGGATGAGAAAGTCGAGT	CCACAACCGTCTTCCACTC
UQCRC	TCCGAGCAGTCCTCTCAG	TCTCAGTCTCAAAACGGCTG
18S	GAGACTCTGGCATGCTAACTAG	GGACATCTAAGGGCATCACAG
TUBULIN	GGCCAGATCTTTAGACCAGAC	CCTTCCGTACCACATCCAG
ACTIN-B	GCACTCTTCCAGCCTTCC	TGTCCACGTCACACTTCATG
RPL19	ATGCCAGAGAAGGTCACATG	ACACATTCCCCTTCACCTTC

The tissue sections were deparaffinized and antigen retrieval was performed at 100 °C for 15 minutes using 0.1M Citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with hydrogen peroxidase 0.3% in PBS for 20 minutes. Sections were incubated at room temperature with monoclonal mouse- anti-human HIF1-α (Abcam, USA (ab8366); 1:1500, diluted in 1% PBSA) overnight. Consecutively, all slides were washed in PBS and incubated with Envision anti-mouse (DAKO, Denmark, Cytomation K4000) for 20 minutes at room temperature. DAB was used for visualization of positively stained breast tissue on the slides and counterstained with haematoxylin, dehydrated and finally mounted with pertex. All slides were stained simultaneously to avoid inter-assay variation. Known highly HIF1-α positive breast tumor tissue served as positive- and a negative-control, the latter was obtained by omitting the primary antibody. Fixed sections were also stained for PKM2 protein expression (Cell Signaling Technology #4053).

Evaluation of immunostaining

Microscopic quantification of HIF1-α-positive breast and/or tumor cells for was performed by two independent, blinded observers (C.E. and T.B.). HIF1-α was scored in the nucleus and cytoplasm, on intensity and percentage, separately. Intensity scores were

as follows: 0 for no staining at all; 1+ for a faint/barely perceptible staining; 2+ for weak to moderate staining; and 3+ for strong staining. Percentage scores were categorized in: 0 for no staining; 1+ for 1-25% of the breast cells/tumor cells stained; 2+ for 26-50% stained and 3+ for ≥50% of the cells stained. For all patients, the product of the intensity score and the percentage score was calculated. Ultimately, the calculated scores for the nucleus and the cytoplasm were summed up for normal and the tumor tissue separately, and dichotomized into low and high expression (<8 and ≥8 for tumor and <6 and ≥6 for the normal tissue) of HIF1-α, based on mean expression scores. PKM2 staining intensity was quantified by two independent observers (S.D. and D.d.V) and dichotomized in to low vs. high expression.

Statistics

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics), Microsoft Excel and Graphpad Prism 6. Patients with missing data were excluded from statistical analyses as it can be assumed that these data were "missing at random". Patients were stratified into two age-groups: 65 to 80 years and ≥ 80 years of age, based on the mean age of the population (mean: 79yrs, range: 65-97yrs). The Mann-Whitney U test was used to evaluate the difference in mRNA expression of the specific primers between young and old patients for the normal and tumor tissue. The χ^2 test was used to evaluate associations between various clinico-pathological parameters and primer specific RT-PCR (dichotomized based on the median value) and immunohistochemical data for the breast tumor and normal breast tissue. The clinical endpoints examined were Overall Survival (OS), defined as the time from date of operation until death by any reason; Disease Free Survival (DFS), defined as date of operation until locoregional recurrence, distant recurrence or breast cancer death (whichever came first), and Relapse Free Period (RFP), defined as date of operation until an event (locoregional recurrence and/or distant recurrence, whichever came first). The Kaplan–Meier method was used to compose survival plots, and the log-rank test was performed for comparison of OS, DFS and RFP curves. Cox Proportional Hazard analyses were used to calculate corresponding Hazard Ratio's (HRs), using univariate analyses. These analyses were additionally adjusted for clinically relevant confounders (normal tissue: number of comorbidities, polypharmacy, dementia, TNM classification and age; for the tumor tissue: latter, plus tumor grade and hormone receptor (HR) status).

RESULTS

Patient and tumor characteristics

One hundred and twenty patients were randomly selected from the original FOCUS cohort (N=3.672). Patients with in situ or metastatic disease, and patients who did not receive breast surgery were excluded. Patient, tumor and treatment characteristics are shown in Table 2. Mean age at diagnosis was 79 years (standard deviation 8.4 years). The majority of patients presented with early stage breast cancer (stage I 30.1%, stage II 51.3%, stage III 15.0%) of ductal morphology (74.1%). No significant associations were seen for mRNA expression in the breast tumor of HIF1-α and its target genes in relation with classic patient and tumor characteristics (Table 3). mRNA expression of these markers in the normal breast tissue showed significant association with age for HIF1-α (p=0.017), GAPDH (p=0.003) and a statistical trend was seen for PKM2 (p=0.069) and VEGFA (p=0.071), all showing higher mRNA expression in the older patients compared to the patients younger than 80 years of age (Table 4). Residing in a nursing home showed a statistical trend with high TFAM mRNA expression (p=0.082) (Table 4). Furthermore, PKM2 (p=0.04), LDHA (p=0.023), COX4 (p=0.035) and UQCRC (p=0.08), all showed significant association with polypharmacy (Table 4). A trend was seen for difficulty with walking and high PKM2 mRNA expression (p=0.066)(Table 4).

High mRNA HIF1-α expression in the normal tissue was significantly associated with the tumor grade of the patient (p=0.045), showing a tendency for higher tumor grades when HIF1-α expression was high in the healthy tissue (Table 4). Lastly, LDHA (p=0.04) and HIF1-α (p=0.024) mRNA expression in the normal tissue were significantly associated with more hormone receptor negative breast tumors (Table 4).

mRNA expression per tissue type

In the normal breast tissue, mRNA expression was significantly higher in the older (≥80 years) compared to the younger patients (65-80 years) for HIF1-α (p=0.0034), GAPDH (p=0.0013), PKM2 (p=0.0135) and VEGFA (p=0.0186) (Figure 1A). Except for a statistical trend for CytB (p=0.0511), we did not observe a significant difference in mitochondrially encoded OXPHOS mRNAs in the normal tissue of the two age groups (Figure 1B). Results showed a non-significant increase in the nuclear encoded OXPHOS mRNAs (COX4 and UQCRC) of the older patients compared to the young.

In the breast tumor tissue no significant difference was seen for the mRNA expression of HIF1-α or any of its targets (Figure 1A). Despite no significant association, we did observe lower expression of the mitochondrially encoded OXPHOS mRNAs (ATP6, COX1 and ND1), with, as is also seen in the healthy breast tissue, an increase of the nuclear encoded OXPHOS mRNAs in the older patients (Figure 1B).

Table 2: Patient and tumor characteristics

	All patients (N=120)	
	N	**%**
Age in years (mean, SD)	79.0 (8.3)	
Number of comorbidities		
0	33	28.4
1	19	16.4
2 or more	64	55.2
Nursing home resident		
No	98	84.5
Yes	18	15.5
Polypharmacy		
No	97	83.6
Yes	19	16.4
Difficulty Walking		
No	97	83.6
Yes	19	16.4
Dementia/Alzheimer		
No	111	95.7
Yes	5	4.3
Tumor stage		
I	34	30.1
II	58	51.3
III	17	15.0
Missing	4	3.5
Tumor grade		
1	20	17.2
2	30	25.9
3	23	19.8
Missing	43	37.1
Tumor morphology		
Ductal	86	74.1
Lobular	16	13.8
Other	14	12.1
HR status		
Negative	26	22.4
Positive	76	65.5
Unknown	14	12.1

Table 3: mRNA expression in tumor tissue and patient and tumor characteristics

	HMOX1			ATP6			COX1			CYTB		
	Low	High		Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
Age												
<80	10 (43.5)	12 (54.5)	0.46	17 (53.1)	14 (43.8)	0.45	15 (48.4)	15 (46.9)	0.90	17 (54.8)	13 (41.9)	0.31
>=80	13 (56.5)	10 (45.5)		15 (46.9)	18 (56.2)		16 (51.6)	17 (53.1)		14 (45.2)	18 (58.1)	
Tumor stage												
1	4 (18.2)	8 (38.1)	0.27	8 (26.7)	11 (34.4)	0.45	7 (24.1)	11 (34.4)	0.80	8 (27.6)	11 (35.5)	0.44
2	15 (68.2)	11 (52.4)		18 (60.0)	16 (50.0)		18 (62.1)	16 (50.0)		17 (58.6)	15 (48.4)	
3	3 (13.6)	1 (4.8)		4 (13.3)	3 (9.4)		3 (10.3)	4 (12.5)		4 (13.8)	3 (9.7)	
Missing	0 (0.0)	1 (4.8)		0 (0.0)	2 (6.2)		1 (3.4)	1 (3.1)		0 (0.0)	2 (6.5)	
Tumor grade												
1	4 (17.4)	3 (13.6)	0.96	10 (31.2)	2 (6.2)	0.06	9 (29.0)	3 (9.4)	0.09	8 (25.8)	4 (12.9)	0.06
2	6 (26.1)	6 (27.3)		8 (25.0)	8 (25.0)		9 (29.0)	7 (21.9)		11 (35.5)	5 (16.1)	
3	4 (17.4)	5 (22.7)		4 (12.5)	8 (25.0)		3 (9.7)	9 (28.1)		3 (9.7)	9 (29.0)	
Missing	9 (39.1)	8 (36.4)		10 (31.2)	14 (43.8)		10 (32.3)	13 (40.6)		9 (29.0)	13 (41.9)	
Tumor morphology												
Ductal	15 (65.2)	17 (77.3)	0.11	27 (84.4)	22 (68.8)	0.32	25 (80.6)	23 (71.9)	0.33	25 (80.6)	22 (71.0)	0.48
Lobular	6 (26.1)	1 (4.5)		3 (9.4)	5 (15.6)		2 (6.5)	6 (18.8)		4 (12.9)	4 (12.9)	
Other	2 (8.7)	4 (18.2)		2 (6.2)	5 (15.6)		4 (12.9)	3 (9.4)		2 (6.5)	5 (16.1)	
ER/PR status												
Negative	7 (30.4)	4 (18.2)	0.40	5 (15.6)	9 (28.1)	0.43	4 (12.9)	9 (28.1)	0.09	4 (12.9)	9 (29.0)	0.27
Positive	16 (69.6)	17 (77.3)		25 (78.1)	22 (68.8)		24 (77.4)	23 (71.9)		25 (80.6)	21 (67.7)	
Unknown	0 (0.0)	1 (4.5)		2 (6.2)	1 (3.1)		3 (9.7)	0 (0.0)		2 (6.5)	1 (3.2)	

p-values calculated with Chi-square test

Abbreviations: ER: estrogen receptor PR: progesterone receptor

ND1			GAPDH			LDHA			PKM1		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
16 (50.0)	14 (45.2)	0.70	13 (41.9)	17 (53.1)	0.37	11 (40.7)	13 (50.0)	0.50	12 (50.0)	9 (37.5)	0.38
16 (50.0)	17 (54.8)		18 (58.1)	15 (46.9)		16 (59.3)	13 (50.0)		12 (50.0)	15 (62.5)	
9 (30.0)	10 (32.3)	0.34	7 (24.1)	11 (34.4)	0.54	7 (26.9)	7 (28.0)	0.76	4 (17.4)	6 (26.1)	0.40
16 (53.3)	17 (54.8)		16 (55.2)	18 (56.2)		15 (57.7)	14 (56.0)		16 (69.6)	12 (52.2)	
5 (16.7)	2 (6.5)		5 (17.2)	2 (6.2)		4 (15.4)	3 (12.0)		3 (13.0)	3 (13.0)	
0 (0.0)	2 (6.5)		1 (3.4)	1 (3.1)		0 (0.0)	1 (4.0)		0 (0.0)	2 (8.7)	
9 (28.1)	3 (9.7)	0.19	8 (25.8)	4 (12.5)	0.33	4 (14.8)	5 (19.2)	0.61	6 (25.0)	2 (8.3)	0.058
9 (28.1)	7 (22.6)		6 (19.4)	9 (28.1)		8 (29.6)	5 (19.2)		5 (20.8)	8 (33.3)	
4 (12.5)	7 (22.6)		4 (12.9)	8 (25.0)		4 (14.8)	7 (26.9)		7 (29.2)	2 (8.3)	
10 (31.2)	14 (45.2)		13 (41.9)	11 (34.4)		11 (40.7)	9 (34.6)		6 (25.0)	12 (50.0)	
26 (81.2)	22 (71.0)	0.62	23 (74.2)	25 (78.1)	0.90	19 (70.4)	19 (73.1)	0.20	19 (79.2)	17 (70.8)	0.36
3 (9.4)	5 (16.1)		4 (12.9)	4 (12.5)		6 (22.2)	2 (7.7)		4 (16.7)	3 (12.5)	
3 (9.4)	4 (12.9)		4 (12.9)	3 (9.4)		2 (7.4)	5 (19.2)		1 (4.2)	4 (16.7)	
4 (12.5)	10 (32.3)	0.16	5 (16.1)	9 (28.1)	0.46	5 (18.5)	8 (30.8)	0.31	4 (16.7)	6 (25.0)	0.24
26 (81.2)	20 (64.5)		24 (77.4)	22 (68.8)		22 (81.5)	17 (65.4)		20 (83.3)	16 (66.7)	
2 (6.2)	1 (3.2)		2 (6.5)	1 (3.1)		0 (0.0)	1 (3.8)		0 (0.0)	2 (8.3)	

PKM2			VEGFA			HIF1A			TFAM		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
16 (53.3)	12 (40.0)	0.30	16 (59.3)	10 (37.0)	0.10	16 (51.6)	15 (45.5)	0.62	13 (48.1)	12 (42.9)	0.69
14 (46.7)	18 (60.0)		11 (40.7)	17 (63.0)		15 (48.8)	18 (54.5)		14 (51.9)	16 (57.1)	
8 (28.6)	7 (23.3)	0.50	10 (37.0)	6 (23.1)	0.45	8 (26.7)	11 (34.4)	0.45	8 (30.8)	9 (33.3)	0.97
14 (50.0)	20 (66.7)		15 (55.6)	15 (57.7)		18 (60.0)	16 (50.0)		14 (53.8)	15 (55.6)	
5 (17.9)	2 (6.7)		2 (7.4)	4 (15.4)		4 (13.3)	3 (9.4)		3 (11.5)	2 (7.4)	
1 (3.6)	1 (3.3)		0 (0.0)	1 (3.8)		0 (0.0)	2 (6.2)		1 (3.8)	1 (3.7)	
8 (26.7)	4 (13.3)	0.27	6 (22.2)	4 (14.8)	0.40	8 (25.8)	4 (12.1)	0.11	6 (22.2)	3 (10.7)	0.41
6 (20.0)	9 (30.0)		9 (33.3)	5 (18.5)		9 (29.0)	7 (21.2)		8 (29.6)	6 (21.4)	
3 (10.0)	7 (23.3)		5 (18.5)	6 (22.2)		7 (22.6)	5 (15.2)		5 (18.5)	5 (17.9)	
13 (43.3)	10 (33.3)		7 (25.9)	12 (44.4)		7 (22.6)	17 (51.5)		8 (29.6)	14 (50.0)	
23 (76.7)	22 (73.3)	0.92	23 (85.2)	19 (70.4)	0.31	25 (80.6)	24 (72.7)	0.13	22 (81.5)	20 (71.4)	0.51
4 (13.3)	4 (13.3)		3 (11.1)	4 (14.8)		5 (16.1)	3 (9.1)		3 (11.1)	3 (10.7)	
3 (10.0)	4 (13.3)		1 (3.7)	4 (14.8)		1 (3.2)	6 (18.2)		2 (7.4)	5 (17.9)	
3 (10.0)	10 (33.3)	0.09	5 (18.5)	9 (33.3)	0.46	5 (16.1)	9 (27.3)	0.45	2 (7.4)	9 (32.1)	0.07
25 (83.3)	19 (63.3)		21 (77.8)	17 (63.0)		25 (80.6)	22 (66.7)		24 (88.9)	18 (64.3)	
2 (6.7)	1 (3.3)		1 (3.7)	1 (3.7)		1 (3.2)	2 (6.1)		1 (3.7)	1 (3.6)	

COX4			UQCRC		
Low	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (75.0)	1 (20.0)	0.09	11 (55.0)	9 (42.9)	0.44
1 (25.0)	4 (80.0)		9 (45.0)	12 (57.1)	
1 (25.0)	1 (20.0)	0.89	6 (30.0)	4 (19.0)	0.59
2 (50.0)	2 (40.0)		11 (55.0)	14 (66.7)	
1 (25.0)	2 (40.0)		3 (15.0)	2 (9.5)	
0 (0.0)	0 (0.0)		0 (0.0)	1 (4.8)	
1 (25.0)	1 (20.0)	0.54	5 (25.0)	3 (14.3)	0.17
0 (0.0)	0 (0.0)		6 (30.0)	4 (19.0)	
1 (25.0)	3 (60.0)		6 (30.0)	4 (19.0)	
2 (50.0)	1 (20.0)		3 (15.0)	10 (47.6)	
3 (75.0)	3 (60.0)	0.638	18 (90.0)	10 (47.6)	**0.01**
1 (25.0)	1 (20.0)		1 (5.0)	6 (28.6)	
0 (0.0)	1 (20.0)		1 (5.0)	5 (23.8)	
0 (0.0)	1 (20.0)	0.34	3 (15.0)	9 (42.9)	0.10
4 (100.0)	4 (80.0)		16 (80.0)	12 (57.1)	
0 (0.0)	0 (0.0)		1 (5.0)	0 (0.0)	

Table 4: mRNA expression in normal breast tissue and patient and tumor characteristics

	HMOX1			VEGFA			PKM2		
	Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
Age									
<80	10 (43.5)	12 (54.5)	0.46	25 (62.5)	16 (42.1)	0.07	28 (63.6)	20 (44.4)	0.07
>=80	13 (56.5)	10 (45.5)		15 (37.5)	22 (57.9)		16 (36.4)	25 (55.6)	
Number of comorbid diseases									
0	6 (26.1)	8 (36.4)	0.59	12 (30.0)	7 (18.4)	0.13	10 (22.7)	14 (31.1)	0.40
1	3 (13.0)	4 (18.2)		4 (10.0)	10 (26.3)		9 (20.5)	5 (11.1)	
2 or more	14 (60.9)	10 (45.5)		24 (60.0)	21 (55.3)		25 (56.8)	26 (57.8)	
Nursing home resident									
No	20 (76.9)	21 (80.8)	0.73	34 (85.0)	33 (86.8)	0.82	41 (93.2)	38 (84.4)	0.19
Yes	6 (23.1)	5 (19.2)		6 (15.0)	5 (13.2)		3 (6.8)	7 (15.6)	
Polypharmacy									
No	22 (84.6)	20 (76.9)	0.48	34 (85.0)	31 (81.6)	0.69	41 (93.2)	35 (77.8)	**0.04**
Yes	4 (15.4)	6 (23.1)		6 (15.0)	7 (18.4)		3 (6.8)	10 (22.2)	
Difficulty walking									
No	21 (80.8)	19 (73.1)	0.51	33 (82.5)	30 (78.9)	0.69	39 (88.6)	33 (73.3)	0.07
Yes	5 (19.2)	7 (26.9)		7 (17.5)	8 (21.1)		5 (11.4)	12 (26.7)	
Dementia / Alzheimer									
No	23 (88.5)	25 (96.2)	0.30	36 (90.0)	37 (97.4)	0.18	43 (97.7)	41 (91.1)	0.18
Yes	3 (11.5)	1 (3.8)		4 (10.0)	1 (2.6)		1 (2.3)	4 (8.9)	
Tumor stage									
1	6 (25.0)	9 (34.6)	0.45	13 (32.5)	7 (18.4)	0.46	14 (32.6)	12 (27.3)	0.90
2	14 (53.8)	13 (50.0)		19 (47.5)	21 (55.3)		22 (51.2)	26 (59.1)	

Table 4: (continued)

	HMOX1			VEGFA			PKM2		
	Low	High		Low	High		Low	High	
	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3	4 (15.4)	4 (15.4)		5 (12.5)	8 (21.1)		6 (14.0)	5 (11.4)	
Missing	2 (7.7)	0 (0.0)		2 (5.0)	2 (5.3)		1 (2.3)	1 (2.3)	
Tumor grade									
1	7 (26.9)	4 (15.4)	0.76	6 (15.0)	9 (23.7)	0.79	9 (20.5)	6 (13.3)	0.44
2	7 (26.9)	8 (30.8)		9 (22.5)	8 (21.1)		14 (31.8)	10 (22.2)	
3	6 (23.1)	6 (23.1)		7 (17.5)	5 (13.2)		6 (13.6)	10 (22.2)	
Missing	6 (23.1)	8 (30.8)		18 (45.0)	16 (42.1)		15 (34.1)	19 (42.2)	
Tumor morphology									
Ductal	18 (69.2)	19 (73.1)	0.67	27 (67.5)	29 (76.3)	0.69	36 (81.8)	30 (66.7)	0.26
Lobular	4 (15.4)	5 (19.2)		7 (17.5)	5 (13.2)		5 (11.4)	9 (20.0)	
Other	4 (15.4)	2 (7.7)		6 (15.0)	4 (10.5)		3 (6.8)	6 (13.3)	
ER/PR status									
Negative	7 (26.9)	6 (23.1)	0.17	7 (17.5)	9 (23.7)	0.68	10 (22.7)	9 (20.0)	0.53
Positive	16 (61.5)	20 (76.9)		28 (70.0)	26 (68.4)		27 (61.4)	32 (71.1)	
Unknown	3 (11.5)	0 (0.0)		5 (12.5)	3 (7.9)		7 (15.9)	4 (8.9)	

p-values calculated with Chi-square test

Abbreviations: ER: estrogen receptor PR: progesterone receptor

PKM1			LDHA			ND1			CYTB		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
20 (57.1)	14 (40.0)	0.15	24 (61.5)	17 (43.6)	0.11	26(52.0)	26 (52.0)	1.0	31 (62.0)	22 (44.0)	0.07
15 (42.9)	21 (60.0)		15 (38.5)	22 (56.4)		24 (48.0)	24 (48.0)		19 (38.0)	28 (56.0)	
6 (17.1)	11 (31.4)	0.24	13 (33.3)	10 (25.6)	0.69	15 (30.0)	14 (28.0)	0.25	11 (22.0)	18 (36.0)	0.30
8 (22.9)	4 (11.4)		5 (12.8)	7 (17.9)		5 (10.0)	11 (22.0)		9 (18.0)	7 (14.0)	
21 (60.0)	20 (57.1)		21 (53.8)	22 (56.4)		30 (60.0)	25 (50.0)		30 (60.0)	25 (50.0)	
31 (88.6)	29 (82.9)	0.50	34 (87.2)	32 (82.1)	0.53	44 (88.0)	42 (84.0)	0.56	44 (88.0)	42 (84.0)	0.56
4 (11.4)	6 (17.1)		5 (12.8)	7 (17.9)		6 (12.0)	8 (16.0)		6 (12.0)	8 (16.0)	
30 (85.7)	28 (80.0)	0.53	37 (94.9)	30 (76.9)	**0.02**	41 (82.0)	43 (86.0)	0.59	42 (84.0)	43 (86.0)	0.78
5 (14.3)	7 (20.0)		2 (5.1)	9 (23.1)		9 (18.0)	7 (14.0)		8 (16.0)	7 (14.0)	
25 (71.4)	30 (85.7)	0.15	31 (79.5)	31 (79.5)	1.0	42 (84.0)	40 (80.0)	0.60	41 (82.0)	40 (80.0)	0.80
10 (28.6)	5 (14.3)		8 (20.5)	8 (20.5)		8 (16.0)	10 (20.0)		9 (18.0)	10 (20.0)	
32 (91.4)	33 (94.3)	0.64	38 (97.4)	36 (92.3)	0.31	49 (98.0)	47 (94.0)	0.31	48 (96.0)	47 (94.0)	0.65
3 (8.6)	2 (5.7)		1 (2.6)	3 (7.7)		1 (2.0)	3 (6.0)		2 (4.0)	3 (6.0)	
12 (35.3)	9 (26.5)	0.66	11 (28.9)	11 (28.9)	0.36	12 (24.5)	15 (30.6)	0.36	17 (34.7)	11 (22.4)	0.21
15 (44.1)	20 (58.8)		19 (50.0)	21 (55.3)		30 (61.2)	22 (44.9)		26 (53.1)	25 (51.0)	

PKM1			**LDHA**			**ND1**			**CYTB**		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
5 (14.7)	4 (11.8)		5 (13.2)	6 (15.8)		5 (10.2)	10 (20.4)		4 (8.2)	11 (22.4)	
2 (5.9)	1 (2.9)		3 (7.9)	0 (0.0)		2 (4.1)	2 (4.1)		2 (4.1)	2 (4.1)	
5 (14.3)	6 (17.1)	0.39	5 (12.8)	8 (20.5)	0.45	6 (12.0)	12 (24.0)	0.46	7 (14.0)	11 (22.0)	0.22
12 (34.3)	7 (20.0)		14 (35.9)	8 (20.5)		15 (30.0)	12 (24.0)		18 (36.0)	9 (18.0)	
7 (20.0)	5 (14.3)		6 (15.4)	8 (20.5)		10 (20.0)	8 (16.0)		8 (16.0)	11 (22.0)	
11 (31.4)	17 (48.6)		14 (35.9)	15 (38.5)		19 (38.0)	18 (36.0)		17 (34.0)	19 (38.0)	
29 (82.9)	22 (62.9)	0.10	29 (74.4)	30 (76.9)	0.94	30 (60.0)	43 (86.0)	**0.01**	36 (72.0)	38 (76.0)	0.82
2 (5.7)	8 (22.9)		5 (12.8)	4 (10.3)		11 (22.0)	4 (8.0)		7 (14.0)	7 (14.0)	
4 (11.4)	5 (14.3)		5 (12.8)	5 (12.8)		9 (18.0)	3 (6.0)		7 (14.0)	5 (10.0)	
7 (20.0)	7 (20.0)	0.75	6 (15.4)	10 (25.6)	**0.04**	13 (26.0)	8 (16.0)	0.23	12 (24.0)	10 (20.0)	0.50
23 (65.7)	25 (71.4)		25 (64.1)	28 (71.8)		30 (60.0)	38 (76.0)		31 (62.0)	36 (72.0)	
5 (14.3)	3 (8.6)		8 (20.5)	1 (2.6)		7 (14.0)	4 (8.0)		7 (14.0)	4 (8.0)	

COX1			ATP6			GAPDH			HIF1A		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
28 (57.1)	24 (49.0)	0.42	29 (56.9)	24 (48.0)	0.37	33 (68.8)	19 (38.8)	**0.003**	33 (64.7)	20 (40.8)	**0.02**
21 (42.9)	25 (51.0)		22 (43.1)	26 (52.0)		15 (31.2)	30 (61.2)		18 (35.3)	29 (59.2)	
15 (30.6)	12 (24.5)	0.51	13 (25.5)	16 (32.0)	0.55	15 (31.2)	13 (26.5)	0.10	12 (23.5)	17 (34.7)	0.43
6 (12.2)	10 (20.4)		7 (13.7)	9 (18.0)		4 (8.3)	12 (24.5)		8 (15.7)	8 (16.3)	
28 (57.1)	27 (55.1)		31 (60.8)	25 (50.0)		29 (60.4)	24 (49.0)		31 (60.8)	24 (49.0)	
45 (91.8)	40 (81.6)	0.14	46 (20.2)	41 (82.0)	0.23	43 (89.6)	41 (83.7)	0.39	44 (86.3)	43 (87.8)	0.83
4 (8.2)	9 (18.4)		5 (9.8)	9 (18.0)		5 (10.4)	8 (16.3)		7 (13.7)	6 (12.2)	
40 (81.6)	42 (85.7)	0.59	41 (80.4)	44 (88.0)	0.30	41 (85.4)	40 (81.6)	0.62	43 (84.3)	41 (83.7)	0.93
9 (18.4)	7 (14.3)		10 (19.6)	6 (12.0)		7 (14.6)	9 (18.4)		8 (15.7)	8 (16.3)	
43 (87.8)	36 (73.5)	0.07	44 (86.3)	38 (76.0)	0.19	40 (83.3)	39 (79.6)	0.64	41 (80.4)	40 (81.6)	0.87
6 (12.2)	13 (26.5)		7 (13.7)	12 (24.0)		8 (16.7)	10 (20.4)		10 (19.6)	9 (18.4)	
48 (98.0)	45 (91.8)	0.17	50 (98.0)	46 (92.0)	0.16	44 (91.7)	48 (98.0)	0.16	47 (92.2)	48 (98.0)	0.18
1 (2.0)	4 (8.2)		1 (2.0)	4 (8.0)		4 (8.3)	1 (2.0)		4 (7.8)	1 (2.0)	
12 (25.0)	16 (33.3)	0.64	15 (30.0)	13 (26.5)	0.55	16 (34.0)	12 (25.0)	0.58	14 (28.0)	14 (29.2)	0.24
28 (58.3)	23 (47.9)		28 (56.0)	24 (49.0)		22 (46.8)	26 (54.2)		24 (48.0)	27 (56.2)	

COX1			ATP6			GAPDH			HIF1A		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
6 (12.5)	8 (16.7)		5 (10.0)	10 (20.4)		8 (17.0)	7 (14.6)		8 (16.0)	7 (14.6)	
2 (4.2)	1 (2.1)		2 (4.0)	2 (4.1)		1 (2.1)	3 (6.2)		4 (8.0)	0 (0.0)	
6 (12.2)	11 (22.4)	0.37	9 (17.6)	9 (18.0)	0.47	9 (16.7)	9 (18.4)	0.97	13 (25.5)	5 (10.2)	**0.05**
16 (32.7)	11 (22.4)		17 (33.3)	10 (20.0)		12 (25.0)	13 (26.5)		13 (25.5)	13 (26.5)	
11 (22.4)	8 (16.3)		8 (15.7)	11 (22.0)		9 (18.8)	10 (20.4)		5 (9.8)	14 (28.6)	
16 (32.7)	19 (38.8)		17 (33.3)	20 (40.0)		19 (39.6)	17 (34.7)		20 (39.2)	17 (34.7)	
32 (65.3)	40 (81.6)	0.15	36 (70.6)	38 (76.0)	0.80	33 (68.8)	(37 (75.5)	0.73	39 (76.5)	34 (69.4)	0.70
9 (18.4)	6 (12.2)		8 (15.7)	7 (14.0)		8 (16.7)	7 (14.3)		7 (13.7)	8 (16.3)	
8 (16.3)	3 (6.1)		7 (13.7)	5 (10.0)		7 (14.6)	5 (10.2)		5 (9.8)	7 (14.3)	
13 (26.5)	8 (16.3)	0.23	11 (21.6)	11 (22.0)	0.29	11 (22.9)	11 (22.4)	0.77	7 (13.7)	15 (30.6)	**0.02**
29 (59.2)	37 (75.5)		32 (62.7)	36 (72.0)		31 (64.6)	34 (69.4)		35 (68.6)	32 (65.3)	
7 (14.3)	4 (8.2)		8 (15.7)	3 (6.0)		6 (12.5)	4 (8.2)		9 (17.6)	2 (4.1)	

TFAM			COX4			UQCRC		
Low	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
11 (52.4)	9 (47.4)	0.75	5 (45.5)	3 (25.0)	0.30	17 (56.7)	13 (41.9)	0.25
10 (47.6)	10 (52.6)		6 (54.5)	9 (75.0)		13 (43.3)	18 (58.1)	
3 (14.3)	5 (26.3)	0.49	3 (27.3)	1 (8.3)	0.32	8 (26.7)	10 (32.3)	0.85
6 (28.6)	3 (15.8)		2 (18.2)	1 (8.3)		5 (16.7)	4 (12.9)	
12 (57.1)	11 (57.9)		6 (54.5)	10 (83.3)		17 (56.7)	17 (54.8)	
19 (90.5)	13 (68.4)	0.08	9 (81.8)	9 (75.0)	0.69	26 (86.7)	26 (83.9)	0.76
2 (9.5)	6 (31.6)		2 (18.2)	3 (25.0)		4 (13.3)	5 (16.1)	
17 (81.0)	17 (89.5)	0.45	11 (100.0)	8 (66.7)	**0.04**	28 (93.3)	24 (77.4)	0.08
4 (19.0)	2 (10.5)		0 (0.0)	4 (33.3)		2 (6.7)	7 (22.6)	
18 (85.7)	14 (73.7)	0.34	9 (81.8)	10 (83.3)	0.92	23 (76.7)	24 (77.4)	0.94
3 (14.3)	5 (26.3)		2 (18.2)	2 (16.7)		7 (23.3)	7 (22.6)	
20 (95.2)	16 (84.2)	0.25	9 (81.8)	12 (100.0)	0.12	28 (93.3)	29 (93.5)	0.97
1 (4.8)	3 (15.8)		2 (18.2)	0 (0.0)		2 (6.7)	2 (6.5)	
8 (40.0)	4 (21.1)	0.61	5 (45.5)	4 (33.3)	0.59	11 (37.9)	9 (30.0)	0.79
10 (50.0)	12 (63.2)		4 (36.4)	7 (58.3)		13 (44.8)	17 (56.7)	

TFAM			COX4			UQCRC		
Low	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
1 (5.0)	2 (10.5)		1 (9.1)	0 (0.0)		3 (10.3)	3 (10.0)	
1 (5.0)	1 (5.3)		1 (9.1)	1 (8.3)		2 (6.9)	1 (3.3)	
3 (14.3)	5 (26.3)	0.29	1 (9.1)	3 (25.0)	0.69	6 (20.0)	8 (25.8)	0.47
5 (23.8)	8 (42.1)		4 (36.4)	5 (41.7)		9 (30.0)	8 (25.8)	
4 (19.0)	2 (10.5)		3 (27.3)	2 (16.7)		4 (13.3)	8 (25.8)	
9 (42.9)	4 (21.1)		3 (27.3)	2 (16.7)		11 (36.7)	7 (22.6)	
12 (57.1)	15 (78.9)	0.22	6 (54.5)	8 (66.7)	0.54	22 (73.3)	24 (77.4)	0.91
5 (23.8)	1 (5.3)		1 (9.1)	2 (16.7)		3 (10.0)	3 (9.7)	
4 (19.0)	3 (15.8)		4 (36.4)	2 (16.7)		5 (16.7)	4 (12.9)	
5 (23.8)	4 (21.1)	0.59	4 (36.4)	3 (25.0)	0.08	5 (16.7)	7 (22.6)	0.49
14 (66.7)	11 (57.9)		4 (36.4)	9 (75.0)		19 (63.3)	21 (67.7)	
2 (9.5)	4 (21.1)		3 (27.3)	0 (0.0.)		6 (20.0)	3 (9.7)	

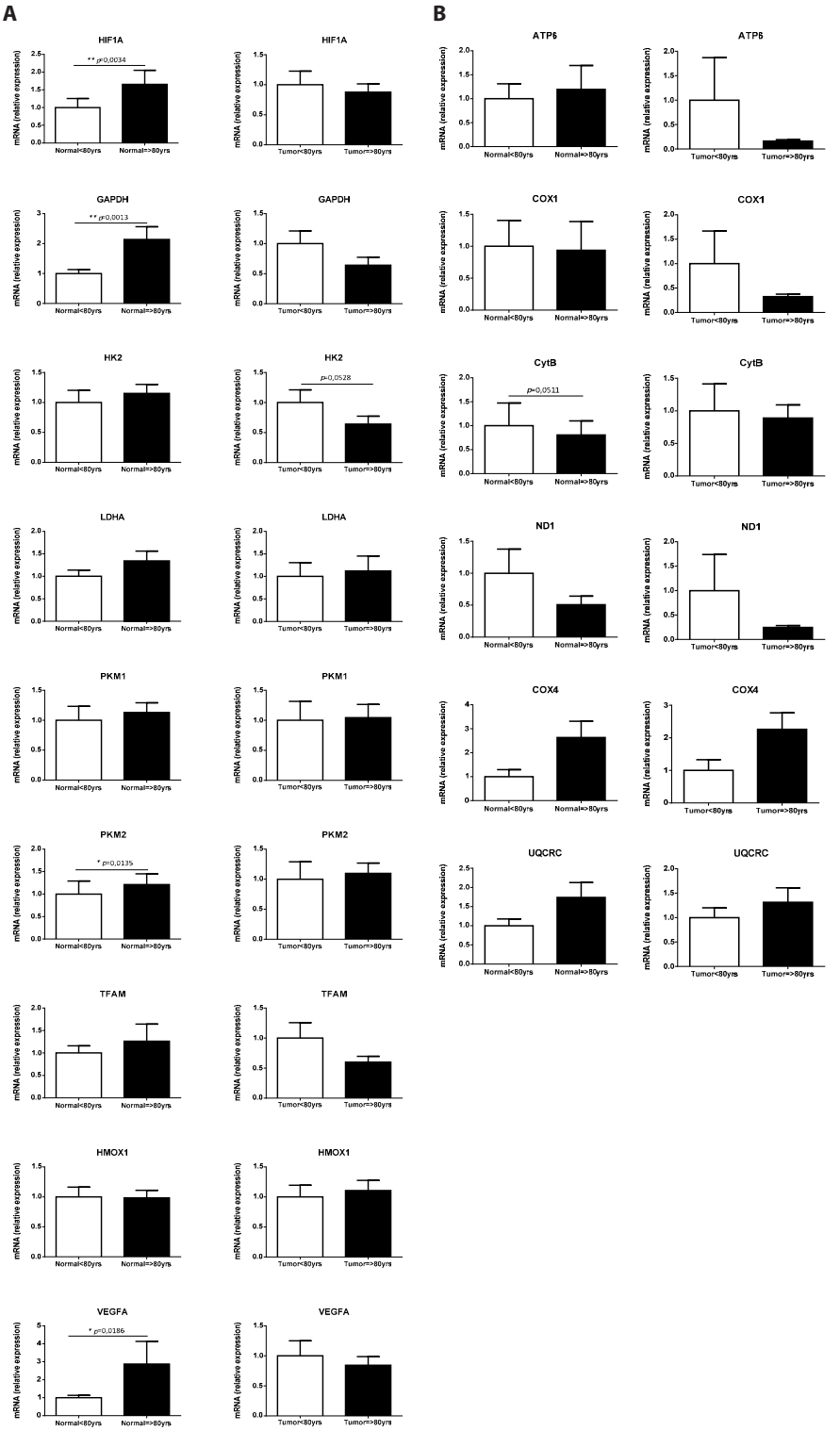

Figure 1A: mRNA expression per primer comparing middle-aged (65-79yrs) and old patients (≥80yrs) in normal breast tissue and breast tumor tissue.

Figure 1B: mRNA expression per mitochondrial gene primer comparing middle-aged (65-79yrs) and old patients (≥80yrs) in normal breast tissue and breast tumor tissue.

mRNA expression per age group

Compared to normal breast tissue, the breast tumor tissue of patients between the ages of 65 and 80 years old, showed a significant increase of HIF1-α (p=0.0011), GAPDH (p=0.0260) and TFAM (p=0.0171) mRNA expression (Figure 2A). This significant increase in the tumor tissue was also seen in patients older than 80 years for HIF1-α (p=0.0242) and TFAM (p=0.0041). A significant decrease was seen in the tumor tissue compared to the healthy breast tissue for the mitochondrially encoded OXPHOS mRNAs, ATP6 (p<0.0001 for age 65-80 years and p<0.0001 for age ≥80 years), COX1 (p=0.0067for age 65-80 years and p=0.0002 for age ≥80 years), and CytB (p=0.0466 for age 65-80 years and p=0.0291 for age ≥80 years)) in both age groups (Figure 2B). For ND1 and both nuclear encoded OXPHOS genes (COX4 and UQCRC) a non-significant decrease was seen.

Clinical outcome

PKM2 expression in the normal breast tissue showed significant association with the OS in the univariate analysis (high vs. low: HR1.71, 95%CI:1.03-2.84, p=0.038) (Table 5A). In the adjusted analysis, the significance was lost (p=0.17) when we included age as an important clinical outcome predictor. However, given the fact that the primary aim of this study was to identify aging markers with clinical value regardless of biological age, given the fact that biological age is often not one-to-one related with physical wellbeing and also given the fact that all of the patients in this cohort are already old, we performed the same adjustment but omitting age as an outcome predictor. Now, a statistical trend was seen in the favour of low PKM2 mRNA expression (HR1.69, 95CI:0.95-3.03, p=0.08, Table 5A). A statistical trend was also seen for high HIF1-α mRNA expression in the normal breast tissue, ignoring age as an outcome predictor (HR1.65, 95%CI:0.77-12.08, p=0.06, Table 5A), and for HMOX1 mRNA expression in the healthy breast tissue, maintaining significance in both forms of adjusted analyses (HR1.95, 95%CI:0.99-3.83, p=0.06 (without age adjustment) and HR1.95, 95%CI:0.98-3.89, p=0.06 (with age adjustment)) (Table 5A).

High PKM2 (HR1.72, 95%CI:0.92-3.22, p=0.087) and VEGFA (HR2.07, 95%CI:1.01-4.14, p=0.039) mRNA expression in the breast tumor tissue were significantly associated with OS in univariate analyses (Table 5B) but lost their significance in the adjusted analyses.

HIF-1α immunohistochemical staining

In normal breast tissue or the breast tumor tissue there was no significant association between HIF1-α and both patient and classical tumor characteristics. In contrast to the qPCR expression data, no significant association was seen for HIF1-α protein expression in the healthy tissue with the OS or in the breast tumor tissue for the OS, DFS and RFP (Table 6).

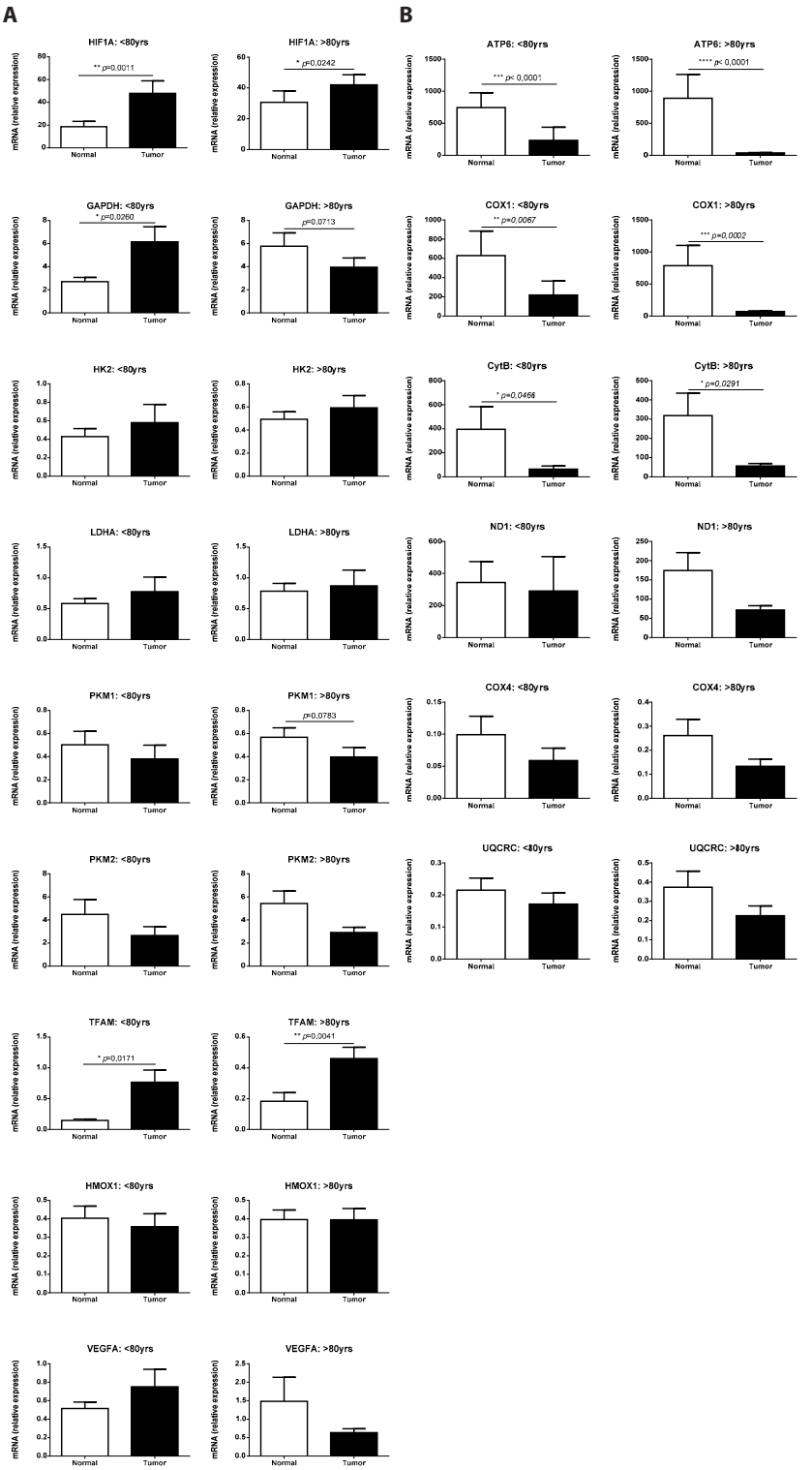

Figure 2A: mRNA expression per primer comparing normal breast tissue and breast tumor tissue in the middle-aged (65-79yrs) and old patients (≥80yrs).

Figure 2B: mRNA expression per mitochondrial gene primer comparing normal breast tissue and breast tumor tissue in the middle-aged (65-79yrs) and old patients (≥80yrs).

Table 5A: Overall Survival (OS) for normal breast tissue

	Univariate survival analyses			Adjusted analyses**			Adjusted analyses*		
	HR	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***
Normal tissue									
VEGFA									
Low	Ref			Ref			Ref		
High	1.49	(0.86-2.57)	0.16	1.27	(0.70-2.28)	0.43	1.17	(0.65-2.13)	0.60
PKM2									
Low	Ref			Ref			Ref		
High	1.71	(1.03-2.84)	**0.04**	1.69	(0.95-3.03)	0.08	2,76	(0.84-2.71)	0.17
PKM1									
Low	Ref			Ref			Ref		
High	1.59	(0.90-2.81)	0.11	1.63	(0.84-3.19)	0.15	1.52	(0.78-2.94)	0.22
LDHA									
Low	Ref			Ref			Ref		
High	1.47	(0.84-2.59)	0.18	1.13	(0.59-2.17)	0.71	1.14	(0.59-2.20)	0.69
HMOX1									
Low	Ref			Ref			Ref		
High	1.44	(0.77-2.68)	0.25	1.95	(0.99-3.83)	0.06	1.95	(0.98-3.89)	0.06
ND1									
Low	Ref			Ref			Ref		
High	1.35	(0.84-2.18)	0.22	1.81	(1.07-3.08)	**0.03**	2.2	(1.26-3.85)	**0.01**
CYTB									
Low	Ref			Ref			Ref		
High	1.50	(0.93-2.43)	0.10	1.84	(1.10-3.10)	**0.02**	1.62	(0.95-2.75)	0.08
COX1									
Low	Ref			Ref			Ref		
High	1.54	(0.95-2.49)	0.08	1.64	(0.98-2.74)	0.06	1.59	(0.95-2.69)	0.08
ATP6									
Low	Ref			Ref			Ref		
High	1.41	(0.88-2.27)	0.15	1.69	(1.01-2.83)	**0.05**	1.64	(0.97-2.77)	0.06
GAPDH									
Low	Ref			Ref			Ref		
High	1.46	(0.89-2.38)	0.13	1.28	(0.75-2.19)	0.36	0.89	(0.49-1.62)	0.72
HIF1A									
Low	Ref			Ref			Ref		
High	1.30	(0.81-2.09)	0.28	1.65	(0.77-12.08)	0.06	1.31	(0.77-2.25)	0.32
TFAM									
Low	Ref			Ref			Ref		
High	0.53	0.26-1.09	0.09	0.50	(0.21-1.23)	0.13	0.52	(0.21-1.27)	0.15
COX4									
Low	Ref			Ref			Ref		
High	1.77	(0.67-4.67)	0.25	1.39	(0.28-7.05)	0.69	0.84	(0.13-5.59)	0.86
UQCRC									
Low	Ref			Ref			Ref		
High	0.91	(0.49-1.69)	0.77	0.76	(0.38-1.54)	0.45	0.62	(0.29-1.32)	0.22

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age
**Not adjusted for age

Table 5B: Overall Survival (OS) for breast tumor tissue

	Univariate survival analyses			Adjusted analyses**			Adjusted analyses*		
	HR	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***	**HR**	**95% C.I.**	***p-value***
Tumor tissue									
ATP6									
Low	Ref			Ref			Ref		
High	1.52	(0.83-2.77)	0.18	1.15	(0.58-2.29)	0.69	0.78	(0.37-1.66)	0.52
COX1									
Low	Ref			Ref			Ref		
High	1.48	(0.80-2.73)	0.21	0.89	(0.42-1.91)	0.77	0.73	(0.33-1.58)	0.42
CYTB1									
Low	Ref			Ref			Ref		
High	2.06	(1.09-3.87)	**0.03**	1.92	(0.84-4.38)	0.12	1.99	(0.83-4.76)	0.12
ND1									
Low	Ref			Ref			Ref		
High	1.31	(0.71-2.41)	0.38	0.87	(0.42-1.84)	0.72	0.79	(0.36-1.72)	0.55
GAPDH									
Low	Ref			Ref			Ref		
High	1.14	(0.62-2.08)	0.68	1.27	(0.61-2.63)	0.53	1.55	(0.70-3.43)	0.28
LDHA									
Low	Ref			Ref			Ref		
High	1.72	(0.89-3.32)	0.11	1.54	(0.70-3.37)	0.28	2.34	(1.01-5.41)	**0.05**
HMOX1									
Low	Ref			Ref			Ref		
High	0.57	(0.27-1.20)	0.14	0.89	(0.35-2.29)	0.81	0.88	(0.34-2.27)	0.79
PKM1									
Low	Ref			Ref			Ref		
High	1.10	(0.57-2.14)	0.77	1.25	(0.53-2.96)	0.61	1.06	(0.45-2.52)	0.90
PKM2									
Low	Ref			Ref			Ref		
High	1.72	(0.92-3.22)	0.09	1.62	(0.76-3.48)	0.21	1.44	(0.65-3.20)	0.37
VEGFA									
Low	Ref			Ref			Ref		
High	2.07	(1.04-4.14)	**0.04**	2.05	(0.70-6.07)	0.19	1.53	(0.47-5.00)	0.49
HIF1A									
Low	Ref			Ref			Ref		
High	1.25	(0.67-2.29)	0.48	1.26	(0.59-2.72)	0.55	1.21	(0.54-2.71)	0.65
TFAM									
Low	Ref			Ref			Ref		
High	1.19	0.63-2.29	0.59	1.19	(0.48-2.94)	0.70	1.03	(0.42-2.54)	0.95
COX4									
Low	Ref			Ref	-		Ref	-	
High	1.58	(0.27-9.12)	0.61	-			-		
UQCRC									
Low	Ref			Ref			Ref		
High	1.46	(0.68-3.12)	0.33	0.95	(0.31-2.91)	0.92	0.87	(0.29-2.62)	0.81

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age,
**Not adjusted for age

Table 6: OS, DFS and RFP for HIF1α and PKM2

	Overall Survival								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
HIF1α Breast tumor tissue									
Low	Ref			Ref			Ref		
High	0.80	(0.48-1.36)	0.42	0.85	(0.44-1.62)	0.61	0.88	(0.44-1.76)	0.72
HIF1α Normal breast tissue									
Low	Ref			Ref			Ref		
High	0.76	(0.47-1.23)	0.27	0.78	(0.47-1.30)	0.33	0.78	(0.46-1.30)	0.33
PKM2 Breast tumor tissue									
Low	Ref			Ref			Ref		
High	1.02	(0.56-1.86)	0.95	1.11	(0.54-2.28)	0.78	0.82	(0.39-1.74)	0.61
PKM2 Normal breast tissue									
Low	Ref			Ref			Ref		
High	1.09	(0.26-4.66)	0.90	57939	(0-∞)	0.97	50155	(0-∞)	0.97

*Adjusted for number of comorbidities, polypharmacy, dementia/Alzheimer's, TNM classification and age (+ tumor grade and HR status in tumor tissue), ** Not adjusted for age

	Disease Free Survival								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
Ref			Ref			Ref		
0.55	(0.18-1.69)	0.30	0.41	(0.11-1.57)	0.19	0.52	(0.13-2.04)	0.35
Ref			Ref			Ref		
1.25	(0.48-3.26)	0.64	1.99	(0.72-5.56)	0.19	2.04	(0.74-5.65)	0.17
Ref			Ref			Ref		
0.19	(0.02-1.46)	0.11	0.10	(0.01-1.17)	***0.07***	0.07	(0.007-0.67)	***0.02***
Ref			Ref			Ref		
22.76	(0-∞)	0.64	64530	(0-∞)	0.98	66055	(0-∞)	0.94

	Relapse Free Period								
	Univariate analyses			Adjusted analyses**			Adjusted analyses*		
HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*	HR	95% C.I.	*p-value*
Ref			Ref			Ref		
0.73	(0.23-2.30)	0.59	0.54	(0.13-2.29)	0.41	0.59	(0.14-2.52)	0.48
Ref			Ref			Ref		
1.15	(0.43-3.08)	0.77	1.37	(0.49-3.85)	0.55	1.30	(0.46-3.67)	0.62
Ref			Ref			Ref		
0.20	(0.03-1.56)	0.12	0.12	(0.01-1.80)	0.12	0.11	(0.01-1.36)	0.09
Ref			Ref			Ref		
22.82	(0-∞)	0.64	60552	(0-∞)	0.98	53578	(0-∞)	0.98

PKM2 immunohistochemical staining

For normal breast tissue and the breast tumor tissue, PKM2 staining showed a significant association with patient and classical tumor characteristics. Tumor tissue showed a statistical trend for DFS, in the favor of high PKM2 staining (HR0.07, 95%CI: 0.007-0.67, p=0.02) (Table 6). A statistical trend, again in the favor of high PKM2 staining in the tumor tissue was seen for RFP (HR0.11, 95%CI: 0.01-1.36, p=0.09). No statistical significance was seen for PKM2 staining in the tumor tissue for OS. No association was seen for PKM2 staining in relation with clinical outcome in the normal breast tissue.

DISCUSSION

This study shows that HIF1-α and its gene targets are upregulated in the healthy breast tissue of older breast cancer patients, not merely closely associated with increased age, but also with surrogate markers of deteriorating clinical functionality of the patient. These include polypharmacy, residing in a nursing home and difficulty walking. Of the investigated markers, PKM2 had the most frequent association with functional surrogate markers, showing a higher expression in the normal breast tissue of the elderly breast cancer population, with a potential negative effect on survival. Furthermore, our results show that HIF1-α expression is significantly higher in the normal breast tissue of the older patient and that HIF1-α expression in the normal breast tissue is associated with a higher tumor grade in the patient. These observations strengthen the hypothesis that dysregulation of the HIF1-α metabolic pathway, presumably leading to an increase in ROS, is closely related with, and maybe even an important driving force of the high cancer incidence in the older population [4].

Given the fact that the adjusted OS analyses for HIF1-α and PKM2 expression in the normal tissue lose their significance when age is considered a confounder (but remains of significant value when age is not taken into account as an outcome predictor) strengthens the observation that these two markers are closely related at an advanced age. Thus, HIF1-α and PKM2 are promising age-related markers, showing a strong association with the patient's clinical condition.

It is already known that cancer cells evolve complex regulatory mechanisms that adapt their metabolism to match physiological states, such as sustained proliferation [18]. Differences in metabolism represent some of the first known variations identified between normal and cancerous cells. A recent study has shown that aging is associated with a decline in nuclear NAD^+ levels, leading to accumulation of HIF1-α under normoxic conditions, paralleling the Warburg effect [12]. It was shown that deletion of the NAD-dependent deacetylase SIRT1 accelerates this process, whereas raising NAD^+ levels in 2

year-old mice restores mitochondrial function to that of a young mouse (3-6 months of age) [12].

Consistent with this, PKM2, an important downstream target of HIF1-α, catalyzing the last step of glycolysis, is upregulated with age and may have important clinical value as an aging marker.

A recent study showed that switching from PKM1 to PKM2 is generated through alternative splicing of two mutually exclusive exons, which is controlled by hnRNPA1, hnRNPA2, and PTB [10]. Given their role in tumorigenesis, hnRNPA1, hnRNPA2 and PTB have potential as therapeutic targets. Promising results are seen with reduction of these proteins using small interfering RNA (siRNA) in cancer cells, leading to cancer tissue specific apoptosis induction [19]. Furthermore, it is proposed that hnRNPA1, A2 and PTB are involved in the early transforming events of tumorigenesis, suggesting that they play an important role in the initial stages of neoplastic transformation [20]. The results of this current study strengthen these previous observations; showing high PKM2 expression in the tissue of the older population, who, based on epidemiological findings, have an increased risk of carcinogenesis and a higher chance of death under these conditions. On the other hand, high PKM2 protein expression in the breast tumor was associated with a significantly better DFS and a trend toward better RFP compared to patients with low PKM2 protein expression in their tumor.

These observations match with the findings of Anastasiou *et al.*, who showed that activation of PKM2 altered cancer metabolism in vitro and reduced xenograft tumor growth [21]. A possible explanation for this finding lies in the fact that highly proliferating cells strongly depend on building blocks, favored by the less active dimeric PKM2. Thus, activation of PKM2 in the active tetrameric form may inhibit cell proliferation due to a deficiency of precursors for the synthesis of cell building blocks [15;21], ultimately leading to less cancer development and spread. PKM2 activation is thus considered a promising adjuvant treatment modality.

The presence of increased HIF1-α and its downstream marker PKM2 in healthy breast tissue are significantly associated with the functional condition of a patient, tumor aggressiveness and clinical outcome. If metabolic changes are important drivers of aging and corresponding tumorigenesis, molecules that prevent, halt or reverse metabolic aging may be useful anti-aging and anti-cancer therapies. Recent promising advances have been made with regard to HIF1-α inhibitors, SIRT activators, PKM2 modulators and NAD-boosting molecules [15;22-24].

A major strength of this study is that, to the best of our knowledge, this is the first study to investigate the hypothesis that metabolic reprogramming in normal tissue during aging correlates with patient and tumor characteristics. However, limitations were the relatively small patient sample size, increasing the chances of underpowered analyses. Second, all study material was formalin fixed paraffin embedded, leading to fragmentation of the RNA. Therefore, primers were designed to obtain small amplicon sizes. Although this did not interfere with our results since qPCR data was always reproduced in a duplicate plate and melting curves were checked for each primer set. Third, considering the hypothesis of this study, it would have been desirable to have younger patients (<65 years), in order to make a clear distinction between the difference in metabolic reprogramming in young versus old. Furthermore, it would have been of value to determine the clinical significance of HIF1-α-induced metabolic reprogramming in healthy tissue of a patient cohort in which survival was not influenced by cancer.

Although, more research is needed to elucidate the potential contribution of age-related changes in HIF1-α and PKM2, the current research supports the hypothesis that reversing or halting metabolic changes during aging could provide considerable benefits to individuals as they reach an age where the chances of tumorigenesis increase exponentially.

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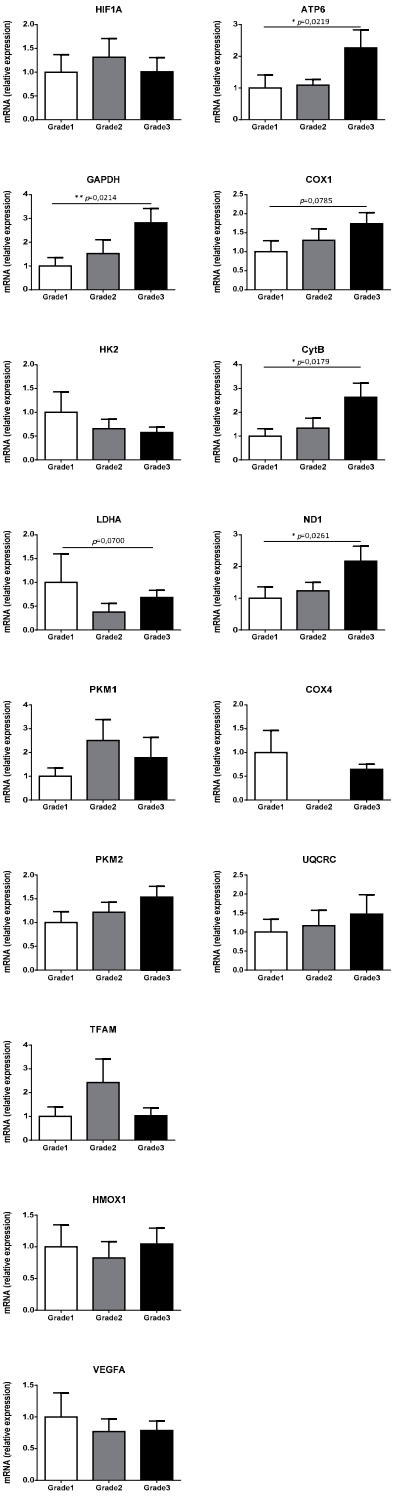
SUPPLEMENTARY TABLE



Extra figure: mRNA expression per primer in the breast tumor tissue per tumor grade.