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Chapter 9

HIF-1α and its metabolic targets are highly expressed in breast tumors of patients of 65 years or older but not in patients younger than 65 years of age

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ABSTRACT

Introduction

HIF-1α is over-expressed in the majority of tumors, taking advantage of HIF-1 α responses including angiogenesis and glycolysis. Accumulating evidence indicates that HIF-1α levels increase during normal aging, a process that is also associated with increased cancer risk. In this study, we investigated the difference in mRNA expression of HIF-1α and its target genes in normal and breast tumor tissue of young and old patients. We hypothesized that HIF-1α plays a more important role in breast cancer development in older than in younger individuals.

Material and Methods

Frozen normal and breast tumor tissue of patients treated at the Leiden University Medical Center (n=35) in the Netherlands were used. Total RNA was extracted from these samples after which quantitative RT-PCR was performed for HIF-1α and its target genes. Expression differences between normal and breast tumor tissue were analyzed per primer, after which patients were stratified in young (<65 years (n=16)) and old (≥65 (n=19)).

Results

Significantly higher HIF-1α (p=0.0097) and associated metabolic, angiogenic (VEGFA: p=0.0311) and inflammatory (HMOX1: p=0.0006) target gene expression was seen in tumors compared to normal breast tissue. In the stratified analysis, the same result was seen for the patients ≥ 65 years but not in patients <65 years.

Conclusion

HIF-1α and its target genes are significantly up-regulated in the tumors of breast cancer patients older than 65 years and less so in patients younger than 65 years indicating that oncologic dysregulation of HIF-1α is more likely to occur in older patients, and that anti-HIF-1α therapy might be an effective therapy for breast cancer patients of a more advanced age.

INTRODUCTION

Hypoxia can occur as a result of a decline in tissue oxygen tension in normal tissues and in different diseases such as vascular and pulmonary disease [1]. In cancer, tumors become hypoxic because of a lack in adequate neovascularisation, often with poor vessel-wall quality. Although hypoxia is toxic for most cells, cancer cells can proliferate in these stressful conditions either by adapting genetically or epigenetically to turn on the hypoxic response pathways. These alterations contribute to the malignant phenotype and behavior of the tumor [1]. The major response to low tissue oxygen levels is mediated by up-regulation of the hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer made up of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. In the absence of oxygen, HIF-1 binds to hypoxia-response elements (HREs), which activates the expression of numerous hypoxia response genes [1]. Target genes of HIF-1 are involved in cell proliferation, angiogenesis (VEGFA, EPO), inflammation (HMOX-1), metabolism (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH), apoptosis, immortalization, and migration [1;2]. In the majority of human cancers, HIF-1α is over-expressed and the tumor cells take advantage of some of these responses, for example, angiogenesis induction and metabolic adaptation, and evade others, such as apoptosis [3]. In previous studies it was shown that HIF-1α overexpression in the tumor is associated with treatment failure and increased mortality [4-6]. Currently, the quest proceeds to develop more efficient anti-cancer strategies, which characterize the products of transcription factor activity essential for tumoregenesis [7]. Based on current knowledge, HIF-1α is nominated as a promising novel therapeutic candidate that fulfills these criteria. However, identification of the cancer patients who would benefit most of this novel therapeutic approach is highly warranted, as such aggressive therapy should not be given to patients who would have minimal benefit of its mode of action.

An important link between aging and cancer was recently demonstrated (Gomes *et al.*,). In old mice, HIF-1α was shown to be stabilized, even in healthy tissue as a consequence of cellular aging, a phenomenon they call "pseudophyopxia" [8].

Their results implied that, during aging, the decline in nuclear nicotinamide adenine dinucleotide (NAD^+) levels, leads to a reduction of Sirtuin 1 (SIRT1) activity in the nucleus, causing Von Hippel-Lindau (VHL) to decline and HIF-1α to be stabilized. This age-induced stabilization of HIF-1α, leads to a pseudohypoxic state that disrupts oxidative phosphorylation (OXPHOS), and promotes a Warburg-like state. The subsequent increase of reactive oxygen species (ROS) may establish an environment for subsequent mutations leading to carcinogenesis, which helps to explain why cancer risk increases exponentially as we age [8;9].

We hypothesize that HIF-1α and its related target genes will be highly expressed and involved in tumorigenesis in older breast cancer populations and less so in younger

counterparts. To test this, we have investigated the difference in expression of HIF-1α and its associated target genes in normal breast tissue and in breast tumor tissue of both young and old patients.

MATERIAL AND METHODS

Patients and tumors

For this study, frozen intra-operative breast tumor and normal breast tissue of patients treated at the Leiden University Medical Center (n=35) in the Netherlands were used. All patients were diagnosed and treated between 2006 and 2014. Twenty-one patients had normal breast tissue samples available and 14 patients had breast tumor tissue available. Thirteen patients had both tissue types available. For all patients the following data was retrieved from the central patient database at the Leiden University Medical Center: age at diagnosis, histological tumor grade (classified as Grade I, II or III) and tumor type (ductal, lobular or "other"), estrogen (ER) and progesterone receptor (PGR) status, human-epidermal growth factor receptor-2 (HER-2) status, pathological tumor and nodal stage, adjuvant treatment received, date of loco-regional/distant recurrence, and date and cause of death if relevant.

Gene expression and mtDNA analysis

Total RNA was extracted from the frozen samples using the miRNeasy extraction mini 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 100ng of RNA. Quantitative 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 18S as an internal control. HIF-1α target genes were chosen based on the most well-known changes occuring in aging and oncogenesis due to HIF-1α stabilization, namely, metabolic adaptation (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH), inflammation (HMOX-1), angiogenesis (VEGFA, EPO), and mitochondrial dysfunction (ATP-6, COX-1, CYTB and ND-1), and were designed using the IDT software. Primer sequences can be found in Table 1.

Statistics

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows, IBM SPSS statistics), Microsoft Excel and Graphpad Prism 6. Hypotheses and analysis plan were drafted before the pathological data was available. Patients with missing RT-PCR data were excluded from statistical analyses as it can be assumed that

these data were "missing at random". The Mann-Whitney U test was used to evaluate the difference in mRNA expression of the specific primers between normal and tumor tissue for the whole cohort, and between normal and tumor tissue for the two age groups (<65 years and ≥65 years). This arbitrary age cut-off was chosen based on epidemiologic literature, in which the age of 65 years is usually considered a cut-off point to identify an elderly population [10]. First, we assessed the difference between primer specific RT-PCR mRNA expression in the two tissue types: normal breast and breast tumor tissue. Next, the same analyses were performed, however patients were now stratified in two age groups, namely, younger than 65 years of age (n=16) and 65 years or older (n=19).

The χ^2 test was used to evaluate associations between various clinico-pathological parameters and primer specific RT-PCR data of the breast tumor tissue.

Table 1: primer sequences used for RT-PCR

		Forward	Reverse
HKG	18S	GAGACTCTGGCATGCTAACTAG	GGACATCTAAGGGCATCACAG
MITO	ATP-6	ACACCCCTTATCCCCATACTAG	ATGGTTGATATTGCTAGGGTGG
MITO	COX-1	GCCATAACCCAATACCAAACG	TTGAGGTTGCGGTCTGTTAG
MITO	CYTB	CAATTATACCCTAGCCAACCCC	GGATAGTAATAGGGCAAGGACG
MITO	ND-1	TCAACCTCAAACTACGCCCTG	GTTGTGATAAGGGTGGAGAGG
HYPOXIA	HIF1α	CCGCTGGAGACACAATCATATC	ACTTCCTCAAGTTGCTGGTC
GLYCO	GLUT-1	TCTGGCATCAACGCTGTCTTC	CGATACCGGAGCCAATGGT
GLYCO	PFKL	GCTGGGCGGCACTATCATT	TCAGGTGCGAGTAGGTCCG
GLYCO	GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG
GLYCO	HK-2	GAGCCACCACTCACCCTACT	CCAGGCATTCGGCAATGTG
GLYCO	LDHA	AGATAAGGAACAGTGGAAAGA	CCAATAGCCCAGGATGTGTAG
GLYCO	PKM-1	ACCGCAAGCTGTTTGAAGAA	TCCATGAGGTCTGTGGAGTG
GLYCO	PKM-2	GAGGCCTCCTTCAAGTGCT	CCAGACTTGGTGAGGACGAT
GLYCO	PDK-1	GGCTGGTTTTGGTTATGGATTG	CTGGGAGTCTTTCTATTGAGTCTG
GLYCO	VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
GLYCO	EPO	TGTGGATAAAGCCGTCAGTG	GGAAGAGTTTGCGGAAAGTG
GLYCO	HMOX1	TCAGGCAGAGGGTGATAGAAG	TTGGTGTCATGGGTCAGC

Abbreviations: HKG: House Keeping Gene MITO: Mitochondrial gene GLYCO: Glycolysis related gene

RESULTS

Patient characteristics

The mean age of this cohort was 62 years (range: 27-91 years). From the 35 samples, 16 samples belonged to patients younger than 65 years and 19 samples were from patients equal to, or older than 65 years. Twenty-one samples (11 samples <65yrs, 10 samples

≥65yrs (mean-age: 59yrs)) were of normal breast histology and 14 samples (5 samples <65yrs, 9 samples ≥65yrs (mean-age: 67yrs)) of breast tumor histology.

Only for ER status a statistical difference was found between patients <65years and patients ≥65years. Conform current practice and observations, significantly more ER positive tumors were seen in the older population (82.4%) compared to the patients younger than 65 years of age (33.3%) (p=0.007, Table 2).

Table 2: tumor and patient characteristics per age group

		<65yrs	≥65yrs	
		n (%)	**n (%)**	***p*-value**
pT				
	1	2 (40.0)	4 (50.0)	0,91
	2	2 (40.0)	3 (37.5)	
	3	1 (20.0)	1 (12.5)	
pN				
	0	2 (40.0)	2 (28.6)	0,83
	1	2 (40.0)	3 (42.9)	
	2	1 (20.0)	1 (14.3)	
	3	0 (0.0)	1 (14.3)	
Tumor grade				
	1	0 (0.0)	0 (0.0)	0,73
	2	2 (40.0)	4 (50.0)	
	3	3 (60.0)	4 (50.0)	
Tumor morphology				
	Ductal	4 (80.0)	6 (66.7)	0,73
	Lobular	0 (0.0)	1 (11.1)	
	Other	1 (20.0)	2 (22.2)	
ER status				
	Negative	3 (60.0)	2 (25.0)	0,21
	Positive	2 (40.0)	6 (75.0)	
PR status				
	Negative	3 (60.0)	3 (37.5)	0,43
	Positive	2 (40.0)	5 (62.5)	

Abbreviations: pT: pathological tumor stage pN: pathological nodal stage ER: estrogen receptor PR: progesterone

Breast cancer vs. normal breast tissue mRNA expression

It was previously reported that cancer is associated with an increase in HIF-1α. Consistent with these reports, we, in this study, show an increase in the HIF-1α mRNA expression in the breast tumor, compared to the normal breast tissue (p=0.0097)(Figure 1). All

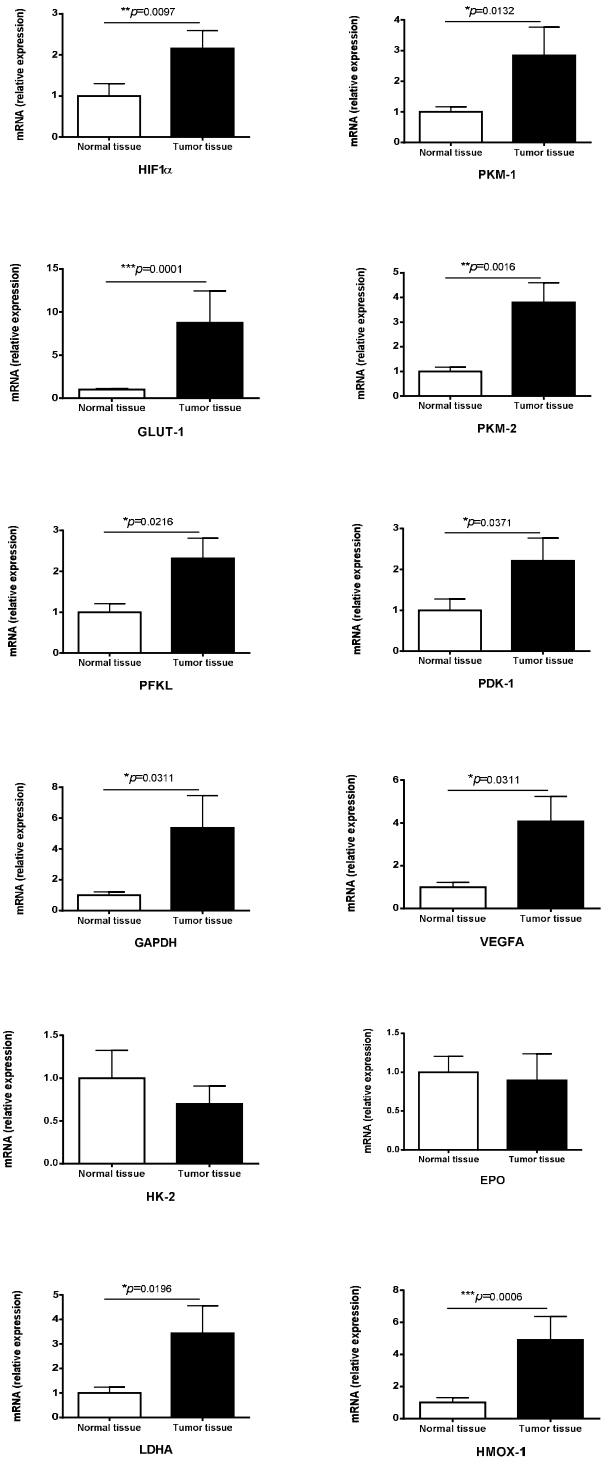

Figure 1:
Column A: Normal breast versus breast tumor mRNA expression per primer.

B

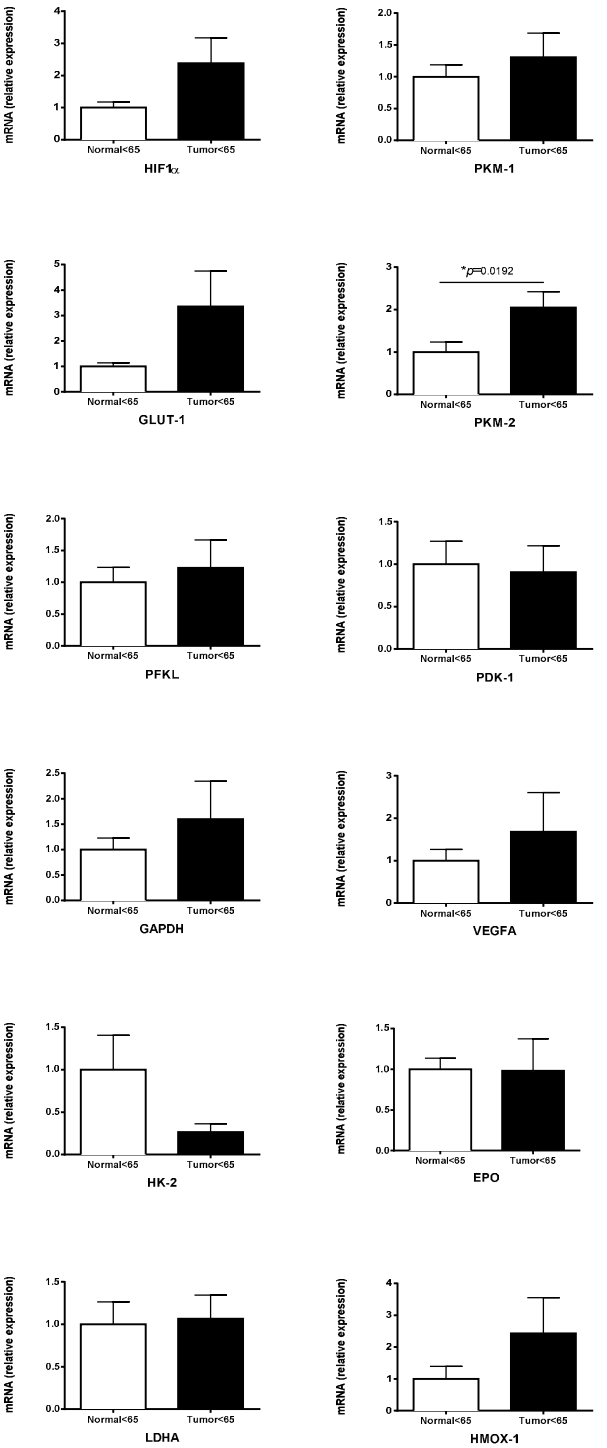

Figure 1:
Column B: Normal breast versus breast tumor mRNA expression per primer for patients younger than 65 years of age.

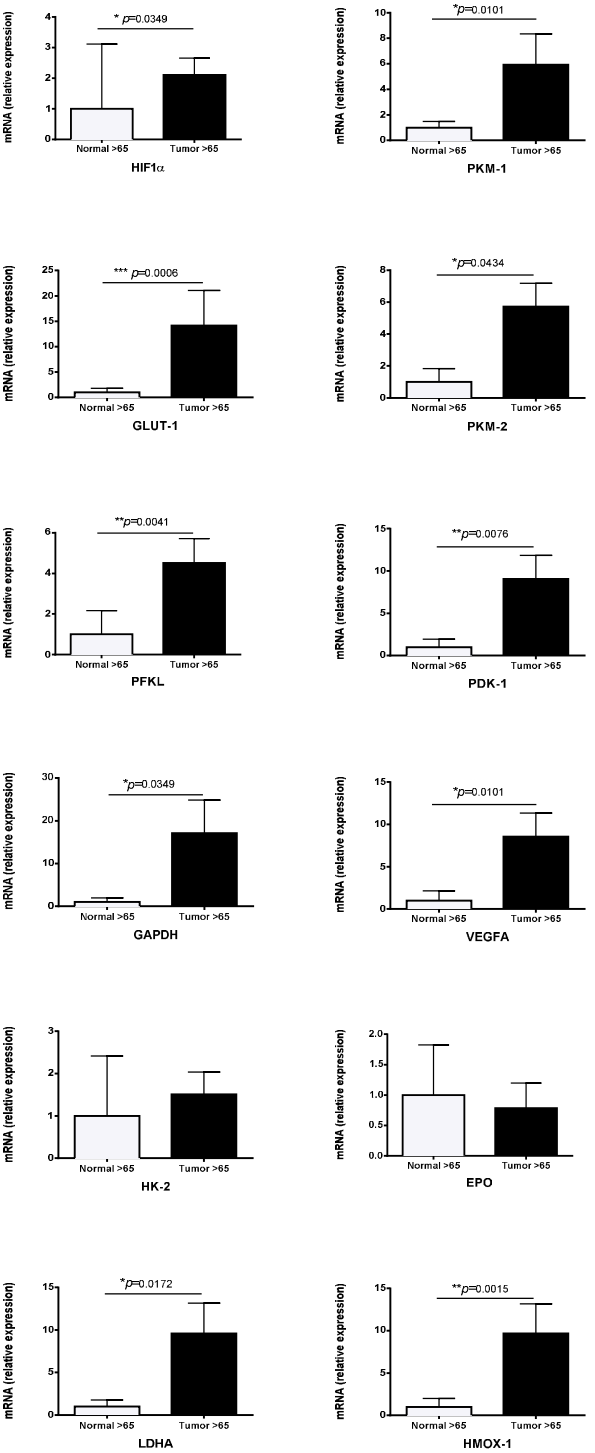

Figure 1:
Column C: Normal breast versus breast tumor mRNA expression per primer for patients ≥65 years of age.

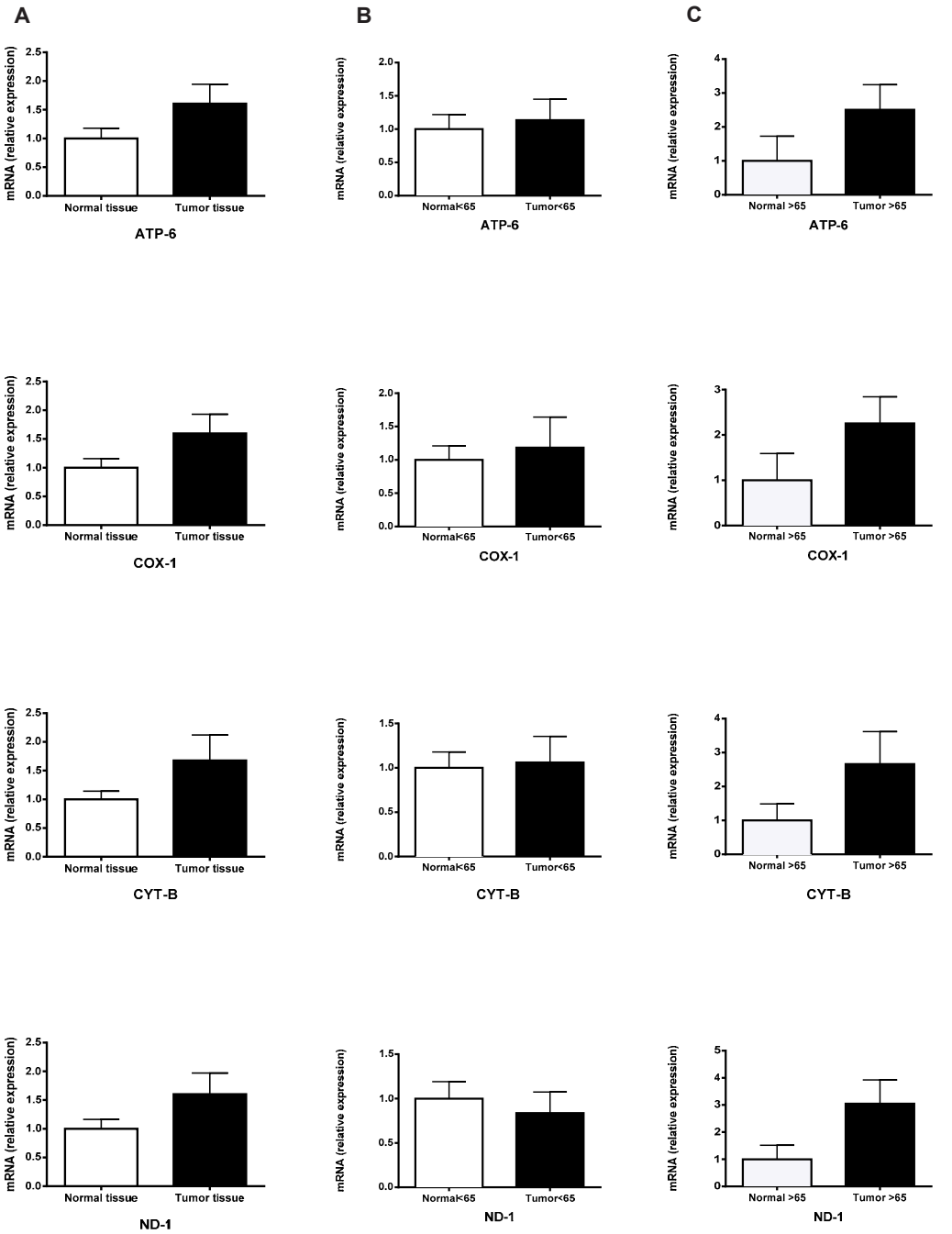

Figure 2:

Column A: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression.

Column B: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression for patients younger than 65 years of age.

Column C: Normal breast versus breast tumor mitochondrially encoded OXPHOS mRNA expression for patients ≥65 years of age.

Table 3: Chi-square associations for patient and tumor characteristics and mRNA expression in the tumor tissue

		ATP6			COX1			CYTB			ND1		
		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													
	<65	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58
	≥65	4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)	
Number of comorbid diseases													
	0	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5
	1	2(28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
	2 or more	2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)	
Tumor stage													
	1	4 (66.7)	2 (28.6)	0,3	3 (50.0)	3 (42.9)	0,15	4 (66.7)	2 (28.6)	0,3	3 (50.0)	3 (42.9)	0,15
	2	1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)	
	3	1 (16.7)	1 (14.3)		2 (33.3)	0 (0.0)		1 (16.7)	1 (14.3)		2 (33.3)	0 (0.0)	
Tumor grade													
	1	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8
	2	3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)	
	3	3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)	
Tumor morphology													
	Ductal	4 (57.1)	6 (85.7)	0,11	5 (71.4)	5 (71.4)	0,51	4 (57.1)	6 (85.7)	0,11	5 (71.4)	5 (71.4)	0,51
	Lobular	0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)	
	Other	3 (42.9)	0 (0.0)		2 (28.6)	1 (14.3)		3 (42.9)	0 (0.0)		2 (28.6)	1 (14.3)	
ER status													
	Negative	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73
	Positive	3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)		3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)	
PR status													
	Negative	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39
	Positive	3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)	

HIF1α			GLUT1			GAPDH			HK2			LDHA		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
2 (28.6)	3 (42.9)	0,58	4 (57.1)	1 (14.3)	0,09	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58
5 (71.4)	4 (57.1)		3 (42.9)	6 (85.7)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)	
1 (14.3)	4 (57.1)	0,17	3 (42.9)	2 (28.6)	0,82	3 (42.9)	2 (28.6)	0,82	2 (28.6)	3 (42.9)	0,5	3 (42.9)	2 (28.6)	0,82
4 (57.1)	1 (14,3)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)	
1 (16.7)	5 (71.4)	0,09	3 (50.0)	3 (42.9)	0,15	3 (50.0)	3 (42.9)	0,15	3 (50.0)	3 (42.9)	0,15	2 (33.3)	4 (57.1)	0,25
3 (50.0)	2 (28.6)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		1 (16.7)	4 (57.1)		2 (33.3)	3 (42.9)	
2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)	
0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,8	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39
3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		3 (50.0)	3 (42.9)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)	
3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)	
4 (57.1)	6 (85.7)	0,42	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06
1 (14.3)	0 (0.0)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)	
2 (28.6)	1 (14.3)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)	
2 (33.3)	3 (42.9)	0,73	2 (33.3)	3 (42.9)	0,73	1 (16.7)	4 (57.1)	0,14	3 (50.0)	2 (28.6)	0,43	2 (33.3)	3 (42.9)	0,73
4 (66.7)	4 (57.1)		4 (66.7)	4 (57.1)		5 (83.3)	3 (42.9)		3 (50.0)	5 (71.4)		4 (66.7)	4 (57.1)	
2 (33.3)	4 (57.1)	0,39	2 (33.3)	4 (57.1)	0,39	1 (16.7)	5 (71.4)	**0,05**	3 (50.0)	3 (42.9)	0,8	2 (33.3)	4 (57.1)	0,39
4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		5 (83.3)	2 (28.6)		3 (50.0)	4 (57.1)		4 (66.7)	3 (42.9)	

PDK1			PFKL			PKM1			PKM2			VEGFA		
Low	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	3 (42.9)	2 (28.6)	0,58	4 (57.1)	1 (14.3)	0,09	3 (42.9)	2 (28.6)	0,58
4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		4 (57.1)	5 (71.4)		3 (42.9)	6 (85.7)		4 (57.1)	5 (71.4)	
1 (14.3)	4 (57.1)	0,22	3 (42.9)	2 (28.6)	0,22	3 (42.9)	2 (28.6)	0,22	3 (42.9)	2 (28.6)	0,82	3 (42.9)	2 (28.6)	0,82
3 (42.9)	2 (28.6)		1 (14.3)	4 (57.1)		1 (14.3)	4 (57.1)		2 (28.6)	3 (42.9)		2 (28.6)	3 (42.9)	
3 (42.9)	1 (14.3)		3 (42.9)	1 (14.3)		3 (42.9)	1 (14.3)		2 (28.6)	2 (28.6)		2 (28.6)	2 (28.6)	
4 (57.1)	2 (33.3)	0,11	4 (66.7)	2 (28.6)	**0,02**	4 (66.7)	2 (28.6)	**0,02**	2 (33.3)	4 (57.1)	0,25	3 (50.0)	3 (42.9)	0,15
1 (14.3)	4 (66.7)		0 (0.0)	5 (71.4)		0 (0.0)	5 (71.4)		2 (33.3)	3 (42.9)		1 (16.7)	4 (57.1)	
2 (28.6)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)		2 (33.3)	0 (0.0)	
0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,8
4 (57.1)	2 (33.3)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)		2 (33.3)	4 (57.1)		3 (50.0)	3 (42.9)	
3 (42.9)	4 (66.7)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)	
6 (85.7)	4 (57.1)	0,42	4 (57.1)	6 (85.7)	0,11	4 (57.1)	6 (85.7)	0,11	4 (57.1)	6 (85.7)	0,11	3 (42.9)	7 (100.0)	0,06
0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		0 (0.0)	1 (14.3)		1 (14.3)	0 (0.0)	
1 (14.3)	2 (28.6)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)		3 (42.9)	0 (0.0)	
2 (28.6)	3 (50.0)	0,43	2 (33.3)	3 (42.9)	0,73	2 (33.3)	3 (42.9)	0,73	3 (50.0)	2 (28.6)	0,43	1 (16.7)	4 (57.1)	0,14
5 (71.4)	3 (50.0)		4 (66.7)	4 (57.1)		4 (66.7)	4 (57.1)		3 (50.0)	5 (71.4)		5 (83.3)	3 (42.9)	
2 (28.6)	4 (66.7)	0,17	2 (33.3)	4 (57.1)	0,39	2 (33.3)	4 (57.1)	0,39	3 (50.0)	3 (42.9)	0,8	1 (16.7)	5 (71.4)	**0,05**
5 (71.4)	2 (33.3)		4 (66.7)	3 (42.9)		4 (66.7)	3 (42.9)		3 (50.0)	4 (57.1)		5 (83.3)	2 (28.6)	

HMOX1			EPO		
Low	**High**		**Low**	**High**	
n (%)	n (%)	*p-value*	n (%)	n (%)	*p-value*
3 (42.9)	2 (28.6)	0,58	2 (33.3)	3 (37.5)	0,87
4 (57.1)	5 (71.4)		4 (66.7)	5 (62.5)	
1 (14.3)	4 (57.1)	0,22	2 (33.3)	3 (37.5)	0,94
3 (42.9)	2 (28.6)		2 (33.3)	3 (37.5)	
3 (42.9)	1 (14.3)		2 (33.3)	2 (25.0)	
3 (42.9)	3 (50.0)	0,34	3 (60.0)	3 (37.5)	0,45
2 (28.6)	3 (50.0)		2 (40.0)	3 (37.5)	
2 (28.6)	0 (0.0)		0 (0.0)	2 (25.0)	
0 (0.0)	0 (0.0)	0,39	0 (0.0)	0 (0.0)	0,43
4 (57.1)	2 (33.3)		3 (60.0)	3 (37.5)	
3 (42.9)	4 (66.7)		2 (40.0)	5 (62.5)	
4 (57.1)	6 (85.7)	0,42	4 (66.7)	6 (75.0)	0,48
1 (14.3)	0 (0.0)		1 (16.7)	0 (0.0)	
2 (28.6)	1 (14.3)		1 (16.7)	2 (25.0)	
2 (28.6)	3 (50.0)	0,43	2 (40.0)	3 (37.5)	0,93
5 (71.4)	3 (50.0)		3 (60.0)	5 (62.5)	
2 (28.6)	4 (66.7)	0,17	2 (40.0)	4 (50.0)	0,73
5 (71.4)	2 (33.3)		3 (60.0)	4 (50.0)	

HIF-1α targets regulating glucose metabolism, except for HK-2, are upregulated in the breast tumor tissue, compared to the normal breast tissue (Figure 1). mRNA expression of *VEGFA* was significantly increased in the tumor tissue compared to the normal tissue (p=0.0311). This was also seen for *HMOX1* (p=0.0006). In contrary to the results presented in the aging study from Gomes *et al.*, in which it was shown that mitochondrially-encoded OXPHOS mRNAs (*ND1, Cytb, COX1 and ATP6*) were significantly lower in aged mice compared to their younger counterparts [8], our data did not show a significant difference between healthy and diseased tissue (Figure 2).

Breast cancer vs. normal breast tissue mRNA expression by age groups

When the cohort was stratified by age, defined as younger than 65 years or 65 years or older, our data showed that HIF-1α was only significantly higher expressed in patients ≥65 years (Figure 1). This was further supported by the significant increase of the HIF-1α targets regulating glucose metabolism (GLUT-1, PFKL, HK-2, PKM-1, PKM-2, LDHA, PDK-1 and GAPDH) in the patients ≥65 years, but not in patients <65 years, except for PKM-2, which showed a significant difference in both age groups (Figure 1). A significant increase was also only seen in the elderly patients for mRNA expression of angiogenesis inducing VEGFA and inflammation regulating HMOX-1.

Although no significance was seen for the comparison of tumor tissue mRNA expression for young and old breast cancer patients, a trend was seen for higher mRNA expression of HIF-1α and its related genes in the old (≥65 years) patients compared to the young (<65 years) (Table 3). We strongly believe that the lack of significance in this analysis can be attributed to the relatively small sample size.

DISCUSSION

Results of this study imply that, in elderly patients, HIF-1α and its targets are significantly up-regulated in breast cancer compared to normal breast tissue. This increase of HIF-1α and its metabolic and angiogenic targets was not seen comparing breast tumor tissue to normal breast tissue of younger patients, even though there was no significant difference in the pathological tumor stage, grade and tumor morphology of the two age groups. It should be noted that a same trend as the ≥ 65 year old cohort, was seen between normal and breast cancer tissue in the younger breast cancer patients, although it was non-significant. These results imply that HIF-1α and its targets certainly play role in tumor development of the younger breast cancer patients, but are less pronounced when compared to the patients above the age of 65 years. This finding indicated that this oncogenic mechanism may be less important in the young. Therefore, it could be postulated that the mode of carcinogenesis is dependent on different mechanisms in the young compared to older breast cancer patients.

The data also supports the possibility that cells from older patients may already be primed high HIF-1α expression due to the so-called age-induced HIF-1α stabilized pseudohypoxic state, as proposed by Gomes *et al.* [8]. This age-related HIF-1α-induced pseudohypoxic state is thought to be responsible for the shift in glucose metabolism primarily performed by the oxygen-dependent tricarboxylic acid (TCA) cycle to glycolysis, the oxygen independent metabolic pathway, also responsible for ATP production [1]. Tumor development, which is known for high HIF-1α expression within the tumor [11], in

an already HIF-1α primed environment, would lead to an additive increase of HIF-1α in the tumor, especially compared to the younger patients.

An emerging paradigm is that the decline in metabolic homeostasis during aging induces a pro-carcinogenic environment, and may be one of the main reasons for the increase in cancer incidence with age [8;9]. We believe that there is also a probability that the significantly higher expression of HIF-1α in the breast tumor compared to the normal tissue of the elderly breast cancer patients plays an important role in the more aggressive, and less therapy-sensitive character of breast cancer in the old, leading to unfavorable outcome in this breast cancer sub-group [12]. In all probability, the mechanism of this more aggressive character lies in the observed metabolic shift, allowing tumor cells to thrive in a low oxygen environment and stimulate angiogenesis [11]. This could also explain the observation of significantly higher VEGF expression in the tumor tissue compared to the normal breast tissue of the older patients, which was not seen in the younger patients.

The ability to survive under hypoxic conditions, characterized by HIF-1α overexpression, is one of the fundamental physiological differences between tumor and healthy cells. Therefore, targeting HIF-1α in the adjuvant setting of cancer treatment has gained substantial ground over the last few years. Particularly, in the older patients, whom are at increased risk of toxicity and adverse events, resulting in non-persistence of the current adjuvant standards, chemotherapy and hormonal therapy [13;14], the demand for targeted therapy increased. In addition, breast cancer mortality increases with age, which may be explained by both undertreatment and overtreatment [12]. New treatment strategies for this group of patients are therefore highly warranted, preferably with a low toxicity profile. Previous studies [15], and also the results of this current study, suggest that HIF-1α overexpression is closely related with increased tumor vascularization, by means of VEGF up-regulation and metabolic adaptation, which in turn is essential for tumor progression. However, only in patients above the age of 65 years the difference in HIF-1α and it's targets expression was significantly different, implying that the greatest effect of HIF-1α blockage will be observed in this sub-group of breast cancer patients. If hypoxia is indeed the reason for the aggressive tumor phenotypes seen in the elderly breast cancer population, blockage of HIF would result in a reduction of tumor growth, due to a disruption in the neovascularization and the metabolic reprogramming, which will subsequently lead to less disease specific mortality in this group of breast cancer patients. Currently, the quest for anti HIF-1α treatment is ongoing. Antisense HIF-1α, such as EZN-2968, by means of down-regulation of HIF-1α and its related genes, was the first molecule studied in hypoxia related gene therapy for cancer [16]. The same research group later reported that expression of the VHL gene suppresses tumor formation by

binding HIF-1α, responsible for stimulating tumor angiogenesis and glycolysis [17]. These results were later also confirmed by Ogura *et al.* [18]. Over the last few years, great improvements have been made on the level of RNA interference techniques, which showed to effectively suppress *in vitro* and *in vivo* growth of hepatobiliary tumors and metastasis, by down-regulating HIF-1α expression [19;20]. Nevertheless, validation of available small molecules in clinical trials to test pharmacological inhibition of the hypoxia-induced pathway is still eagerly awaited. In summary, this very promising novel pharmacologic approach to cancer therapy will, based on the expression profiles presented in this current study, be of special interest for the elderly breast cancer patients, as they present themselves as the breast cancer sub-population who could potentially optimally benefit from this specific pathway blockage.

This is the first study to assess the relationship between HIF-1α and its downstream genes in normal and the cancerous tissue of young and elderly patients with breast cancer. This study, however, also has its limitations. First, this study group did not contain enough events (death or relapse) to reliably report on the clinical significance of the expression of HIF-1α and its related markers in the two age groups. Furthermore, the overall patient count is small, resulting in a lack of power to show significant associations in the chi-square tests performed for the mRNA expression per marker for the different age groups in the breast tumor tissue. Even though the registered values in table 3 indicate high probability of mRNA expression difference between the two age groups.

In conclusion, this work shows that HIF-1α and its related genes are significantly up-regulated in the tumor tissue of breast cancer patients 65 years or older but not in patients younger than 65 years, consistent with HIF-1a playing a more critical role in the tumors of elderly patients and providing evidence for the geroncogenesis theory [9]. Although more work is necessary to verify our findings, these results indicate that small compounds that prevent HIF-1α stabilization or promote its degradation might be an effective therapy particularly for breast cancer patients of a more advanced age.

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