Determining the significance of the expression of variant erythropoietin receptor forms in human breast cancer

Final report

Anemia is present in many cancer patients at the time of diagnosis and/or as the result of cancer therapy[1-4]. Anemia has been found not only to impair the quality of life, but also to reduce the duration of survival and lead to poor clinical outcome[1-3]. In cancer patients, anemia has been shown to lead to tumor hypoxia, angiogenesis, and resistance to chemo- and radiotherapy[1,5-8]. Because of the apparent interconnections between anemia, hypoxia, tumor responsiveness to therapy and outcomes, clinical studies have been conducted in the assumption that correction of anemia will not only alleviate anemia-related symptoms but also improve tumor response to therapy and extend overall survival time[2,9-10]. Iron supplementation, blood transfusion, erythropoesis stimulating agents (ESAs) or the combination of these are used in the clinical settings for correction of anemia.

ESAs are recombinant glycoproteins that stimulate red-blood-cell production using the same molecular mechanism as endogenous erythropoietin[11]. While ESAs treatment was clearly shown to be effective in reducing transfusion requirements and improve quality of life[2,4,9-10], clinical trials suggested a potential adverse effect of ESAs on tumor recurrence and patient survival, and it was shown that ESAs administration is associated with an increase in thromboembolic events[12-13]. The US Food and Drug Administration boxed warning was issued for erythropoietin-stimulating agents regarding serious adverse events in March 2007[14]. Although the warning was effective in reducing the utilization of ESAs, the effect of ESAs treatment remained inconclusive according to the clinical trials[15]. Furthermore, after the 11 years from the black box warning issued by FDA the clear molecular evidence about the mechanism(s) by ESAs might affect survival or stimulate tumor progression is still missing.

Studies presented that ESAs may enhance cancer cell migration[10], invasiveness[10,16-17], survival[17-23] and proliferation[24-27] of tumors, but a defined biological mechanism(s) for ESAs/EpoR signaling in cancer cells is still controversial.

EpoR signaling is known to rely on a delicate structural regulation of the protein and even a single amino acid change can result in constitutive activation of the receptor[28]. Interestingly, the fact that several EpoR splice variants have been described in both normal brain[29] and malignant tumors[14] has been completely overlooked in studies examining the role of EpoR in cancer.

Previously, using quantitative RT-PCR we measured the expression levels of all eight exons of the EpoR gene in cancer cell lines and found that mRNA regions coding for the extracellular portion of the receptor are expressed at a significantly lower levels compared to the C-terminal region in breast and ovarian cancer cells. In contrast, no such difference was seen in UT-7 cells which known to express functional, wild type EpoR. Based on our previous results, we hypothesized that different erythropoietin receptor variant forms exist in human breast cancer. These altered forms of EpoR may be responsible for the observed differences in the Epo responsiveness of EpoR bearing breast cancers and might responsible for the inconsistency of the published data with functional role of EpoR expression in tumor cells and cell-lines. The aim of the project was to characterize and examine the role of the EpoR variant forms in breast carcinomas using different molecular biological approaches.

Materials and methods:

RNA preparation and Quantitative Real-Time RT-PCR Assays

For RNA preparation 189 microdissected primary breast samples were subjected according to manufacture's instructions (Qiagen, GmbH, Germany). Reverse transcription (RT) was carried out from 600 ng total RNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor, according to the protocol of the supplier (Applied Biosystems). Predesigned Taqman Gene Expression Assay (Applied Biosystems) for EpoR 2-3 exon (Hs00959427_m1), EpoR 7-8 exon (Hs00959432_m1) were applied to detect of EpoR mRNA expression. Because the normalization of the gene expression data is more accurate if we apply multiple house-keeping genes[30], geometric mean of GAPDH (Hs99999905_m1) and ACHBT (Hs01060665_g1) were used as controls. For all qPCR reactions standard concentration of assays and Universal TaqMan PCR mastermix were applied, according to manufacture's instructions (Applied Biosystems).

Applied Biosystems® TaqMan® Array Human Angiogenesis assays were applied to investigate the expression of angiogenesis and lymphangiogenesis associated genes according to the manufacture's instructions (Applied Biosystems) in the case of 34 selected samples. The 96-well Plate contains 92 genes which are involved in angiogenesis and lymphangiogenesis process and 4 endogenous control genes (Table 1).

The data analysis was performed in an Excel datasheet.

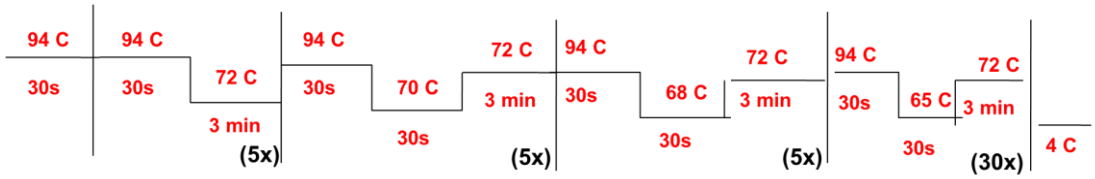
1. Table: List of genes of Human Angiogenesis assays

Well Position	Assay ID	Gene Symbol
A01	Hs99999901_s1	18S
A02	Hs99999905_m1	GAPDH
A03	Hs99999909_m1	HPRT1
A04	Hs99999908_m1	GUSB
A05	Hs00241027_m1	FGA
A06	Hs00264877_m1	PLG
A07	Hs00166654_m1	SERPINC1
A08	Hs00168730_m1	PRL
A09	Hs00234422_m1	MMP2
A10	Hs02379000_s1	ANG,RNASE4
A11	Hs00181613_m1	ANGPT1
A12	Hs00169867_m1	ANGPT2
B01	Hs00171022_m1	CXCL12
B02	Hs00174781_m1	EDIL3
B03	Hs00362096_m1	EPHB2
B04	Hs00265254_m1	FGF1
B05	Hs00266645_m1	FGF2
B06	Hs00173564_m1	FGF4
B07	Hs00246256_m1	FST
B08	Hs00300159_m1	HGF
B09	Hs00174103_m1	IL8
B10	Hs00174877_m1	LEP
B11	Hs00171064_m1	MDK
B12	Hs00157317_m1	TYMP
C01	Hs00234042_m1	PDGFB
C02	Hs00383235_m1	PTN
C03	Hs00260905_m1	PROK1
C04	Hs00608187_m1	TGFA
C05	Hs99999918_m1	TGFB1
C06	Hs00174128_m1	TNF
C07	Hs00900054_m1	VEGFA
C08	Hs00173634_m1	VEGFB
C09	Hs00153458_m1	VEGFC
C10	Hs00170014_m1	CTGF
C11	Hs00197064_m1	FBLN5
C12	Hs00962914_m1	THBS1
D01	Hs00270802_s1	TNFSF15
D02	Hs00168433_m1	ITGA4
D03	Hs01077958_s1	IFNB1
D04	Hs00174143_m1	IFNG
D05	Hs00171042_m1	CXCL10
D06	Hs00168405_m1	IL12A
D07	Hs00171467_m1	SERPINF1
D08	Hs00427220_g1	PF4
D09	Hs00208609_m1	VASH1
D10	Hs00199608_m1	ADAMTS1
D11	Hs00559786_m1	ANGPTL1
D12	Hs00611096_m1	AMOT
E01	Hs00153304_m1	CD44
E02	Hs00174344_m1	CDH5
E03	Hs00601975_m1	CXCL2
E04	Hs00184728_m1	SERPINB5
E05	Hs00176573_m1	FLT1

E06	Hs00188273_m1	SEMA3F
E07	Hs00176096_m1	TEK
E08	Hs00178500_m1	TIE1
E09	Hs00223332_m1	TNMD
E10	Hs00234278_m1	TIMP2
E11	Hs00165949_m1	TIMP3
E12	Hs00765775_m1	ANGPTL2
F01	Hs00205581_m1	ANGPTL3
F02	Hs00236077_m1	CEACAM1
F03	Hs00232618_m1	HEY1
F04	Hs00233808_m1	ITGAV
F05	Hs00169777_m1	PECAM1
F06	Hs00272659_m1	LYVE1
F07	Hs00174029_m1	KIT
F08	Hs00913333_m1	TNNI1
F09	Hs00187290_m1	NRP2
F10	Hs00176676_m1	KDR
F11	Hs00196470_m1	ENPP2
F12	Hs00189521_m1	FIGF
G01	Hs00270951_s1	FOXC2
G02	Hs00266237_m1	COL4A1
G03	Hs01098873_m1	COL4A2
G04	Hs00266332_m1	COL15A1
G05	Hs00194179_m1	HSPG2
G06	Hs00181017_m1	COL18A1
G07	Hs01549940_m1	FN1
G08	Hs01022527_m1	COL4A3
G09	Hs01011995_g1	F2
G10	Hs01105174_m1	BAI1
G11	Hs00900373_m1	CHGA
G12	Hs00211115_m1	ANGPT4
H01	Hs99999083_m1	CSF3
H02	Hs00963711_g1	GRN
H03	Hs01568063_m1	THBS2
H04	Hs00993254_m1	LECT1
H05	Hs01101127_m1	ANGPTL4
H06	Hs01001469_m1	ITGB3
H07	Hs00998026_m1	PDGFRA
H08	Hs00387364_m1	PDGFRB
H09	Hs01047677_m1	FLT4
H10	Hs00826128_m1	NRP1
H11	Hs01922614_s1	S1PR1
H12	Hs00896294_m1	PROX1

Smart Race PCR and sequence analysis

In order to identify complete variant EpoR mRNA forms from cytosolic RNA we have applied SMART RACE PCR (Switching Mechanism At 5' end of RNA Transcript and Rapid Amplification of cDNA Ends, Clontech, Mountain View, CA, USA) assay using
EpoR specific primer sets (forward 5'CCT GAC GCT CTC CCT CAT CC 3'; reverse 5'GCC TTC AAA CTC GCT CTC TGG 3'). The PCR cycle protocol for the EPOR was the following:



The products of the SMART RACE PCR reactions were separated by agarose gel electrophoresis. Bands were visualized by the AlphaImager 3300 Imaging System (Alpha Innotech, San Leandro, CA). DNA was purified from the individual bands and subjected to EpoR specific TaqMan analysis to verify the presence of EpoR sequences in the SMART RACE PCR products. After the verification the purified SMART RACE PCR products were subjected to sequence analysis after automated sequencing. For the sequence analysis Splign software was used (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi) with applying the following EpoR sequence:

NM_000121.2 Full length EpoR sequence:

ACTTAGAGGCGCCTGGTCGGGAAGGGCCTGGTCAGCTGCGTCCGGCGGAGGCAGCTGCTGACCCAGCTGTGGACT
GTGCCGGGGGCGGGGGACGGAGGGGCAGGAGCCCTGGGCTCCCCGTGGCGGGGGCTGTATCATGGACCACCTCGG
GGCGTCCCTCTGGCCCCAGGTCGGCTCCCTTTGTCTCCTGCTCGCTGGGGCCGCCTGGGCGCCCCCGCCTAACCT
CCCGGACCCCAAGTTCGAGAGCAAAGCGGCCTTGCTGGCGGCCCGGGGGCCCGAAGAGCTTCTGTGCTTCACCGA
GCGGTTGGAGGACTTGGTGTGTTTCTGGGAGGAAGCGGCGAGCGCTGGGGTGGGCCCGGGCAACTACAGCTTCTC
CTACCAGCTCGAGGATGAGCCATGGAAGCTGTGTCGCCTGCACCAGGCTCCCACGGCTCGTGGTGCGGTGCGCTT
CTGGTGTTCGCTGCCTACAGCCGACACGTCGAGCTTCGTGCCCCTAGAGTTGCGCGTCACAGCAGCCTCCGGCGC
TCCGCGATATCACCGTGTCATCCACATCAATGAAGTAGTGCTCCTAGACGCCCCCGTGGGGCTGGTGGCGCGGTT
GGCTGACGAGAGCGGCCACGTAGTGTTGCGCTGGCTCCCGCCGCCTGAGACACCCATGACGTCTCACATCCGCTA
CGAGGTGGACGTCTCGGCCGGCAACGGCGCAGGGAGCGTACAGAGGGTGGAGATCCTGGAGGGCCGCACCGAGTG
TGTGCTGAGCAACCTGCGGGGCCGGACGCGCTACACCTTCGCCGTCCGCGCGCGTATGGCTGAGCCGAGCTTCGG
CGGCTTCTGGAGCGCCTGGTCGGAGCCTGTGTCGCTGCTGACGCCTAGCGACCTGGACCCCCTCATCCTGACGCT
CTCCCTCATCCTCGTGGTCATCCTGGTGCTGCTGACCGTGCTCGCGCTGCTCTCCCACCGCCGGGCTCTGAAGCA
GAAGATCTGGCCTGGCATCCCGAGCCCAGAGAGCGAGTTTGAAGGCCTCTTCACCACCCACAAGGGTAACTTCCA
GCTGTGGCTGTACCAGAATGATGGCTGCCTGTGGTGGAGCCCCTGCACCCCCTTCACGGAGGACCCACCTGCTTC
CCTGGAAGTCCTCTCAGAGCGCTGCTGGGGGACGATGCAGGCAGTGGAGCCGGGGACAGATGATGAGGGCCCCCT
GCTGGAGCCAGTGGGCAGTGAGCATGCCCAGGATACCTATCTGGTGCTGGACAAATGGTTGCTGCCCCGGAACCC
GCCCAGTGAGGACCTCCCAGGGCCTGGTGGCAGTGTGGACATAGTGGCCATGGATGAAGGCTCAGAAGCATCCTC

CTGCTCATCTGCTTTGGCCTCGAAGCCCAGCCCAGAGGGAGCCTCTGCTGCCAGCTTTGAGTACACTATCCTGGA
CCCCAGCTCCCAGCTCTTGCGTCCATGGACACTGTGCCCTGAGCTGCCCCCTACCCCACCCCACCTAAAGTACCT
GTACCTTGTGGTATCTGACTCTGGCATCTCAACTGACTACAGCTCAGGGGACTCCCAGGGAGCCCAAGGGGGCTT
ATCCGATGGCCCCTACTCCAACCCTTATGAGAACAGCCTTATCCCAGCCGCTGAGCCTCTGCCCCCCAGCTATGT
GGCTTGCTCTTAGGACACCAGGCTGCAGATGATCAGGGATCCAATATGACTCAGAGAACCAGTGCAGACTCAAGA
CTTATGGAACAGGGATGGCGAGGCCTCTCTCAGGAGCAGGGGCATTGCTGATTTTGTCTGCCCAATCCATCCTGC
TCAGGAAACCACAACCTTGCAGTATTTTTAAATATGTATAGTTTTTTTG

EpoR CDNS:

ATGGACCACCTCGGGGCGTCCCTCTGGCCCCAGGTCGGCTCCCTTTGTCTCCTGCTCGCTGGGGCCGCCT
GGGCGCCCCCGCCTAACCTCCCGGACCCCAAGTTCGAGAGCAAAGCGGCCTTGCTGGCGGCCCGGGGGCC
CGAAGAGCTTCTGTGCTTCACCGAGCGGTTGGAGGACTTGGTGTGTTTCTGGGAGGAAGCGGCGAGCGCT
GGGGTGGGCCCGGGCAACTACAGCTTCTCCTACCAGCTCGAGGATGAGCCATGGAAGCTGTGTCGCCTGC
ACCAGGCTCCCACGGCTCGTGGTGCGGTGCGCTTCTGGTGTTCGCTGCCTACAGCCGACACGTCGAGCTT
CGTGCCCCTAGAGTTGCGCGTCACAGCAGCCTCCGGCGCTCCGCGATATCACCGTGTCATCCACATCAAT
GAAGTAGTGCTCCTAGACGCCCCCGTGGGGCTGGTGGCGCGGTTGGCTGACGAGAGCGGCCACGTAGTGT
TGCGCTGGCTCCCGCCGCCTGAGACACCCATGACGTCTCACATCCGCTACGAGGTGGACGTCTCGGCCGG
CAACGGCGCAGGGAGCGTACAGAGGGTGGAGATCCTGGAGGGCCGCACCGAGTGTGTGCTGAGCAACCTG
CGGGGCCGGACGCGCTACACCTTCGCCGTCCGCGCGCGTATGGCTGAGCCGAGCTTCGGCGGCTTCTGGA
GCGCCTGGTCGGAGCCTGTGTCGCTGCTGACGCCTAGCGACCTGGACCCCCTCATCCTGACGCTCTCCCT
CATCCTCGTGGTCATCCTGGTGCTGCTGACCGTGCTCGCGCTGCTCTCCCACCGCCGGGCTCTGAAGCAG
AAGATCTGGCCTGGCATCCCGAGCCCAGAGAGCGAGTTTGAAGGCCTCTTCACCACCCACAAGGGTAACT
TCCAGCTGTGGCTGTACCAGAATGATGGCTGCCTGTGGTGGAGCCCCTGCACCCCCTTCACGGAGGACCC
ACCTGCTTCCCTGGAAGTCCTCTCAGAGCGCTGCTGGGGGACGATGCAGGCAGTGGAGCCGGGGACAGAT
GATGAGGGCCCCCTGCTGGAGCCAGTGGGCAGTGAGCATGCCCAGGATACCTATCTGGTGCTGGACAAAT
GGTTGCTGCCCCGGAACCCGCCCAGTGAGGACCTCCCAGGGCCTGGTGGCAGTGTGGACATAGTGGCCAT
GGATGAAGGCTCAGAAGCATCCTCCTGCTCATCTGCTTTGGCCTCGAAGCCCAGCCCAGAGGGAGCCTCT
GCTGCCAGCTTTGAGTACACTATCCTGGACCCCAGCTCCCAGCTCTTGCGTCCATGGACACTGTGCCCTG
AGCTGCCCCCTACCCCACCCCACCTAAAGTACCTGTACCTTGTGGTATCTGACTCTGGCATCTCAACTGA
CTACAGCTCAGGGGACTCCCAGGGAGCCCAAGGGGGCTTATCCGATGGCCCCTACTCCAACCCTTATGAG
AACAGCCTTATCCCAGCCGCTGAGCCTCTGCCCCCCAGCTATGTGGCTTGCTCTTAG

For the sequencing, EpoR ISO1 assay (IDT) was used:

qPCR upper primer 1-19 5'-ATTCCGCCCCCAGGTGGAG-3'
qPCR probe 5'- CAGCACACACTCGGTGCGGCCCTCCAGGAT-3'
qPCR lower 72-91 5'- GCGGACGGCGAAGGTGTAGC-3'
For EpoR primers are Tm 62-63, probe is Tm 71

Cell culture

Human breast cancer cell lines MDA-MB-436, MDA-MB 231, MDA-MB-468, T47D and SKBR3 were maintained in RPMI medium supplemented with 10% fetal bovine serum, L-

glutamine and antibiotics. All chemicals were of reagent grade and obtained from Sigma-Aldrich (Sigma-Aldrich, Budapest, Hungary). Media of UT-7 cells were supplemented with 10 U/ml rHuEpo (Epogen, Epoetin; Amgen Pharmaceuticals, Thousand Oaks, CA) after each passage. To test the short-term effect of rHuEpo treatment for the EpoR expression on cell lines, subconfluent cultures of HS5784, MDA-MB436, MDA-MB435, MDA-MB231, MDA-MB468, MCF7, T47D, B1549 and SKBR3 cells were treated for 5 minutes with rHuEpo in six-well plates after overnight serum (1% FBS) starvation.

shRNA plasmids

The pBCMGS-Neo expression vector containing human wild-type full-length EpoR was generously provided by Dr. Ács [31]. Plasmids were nucleofected into MDA-MB 436 cells using an Amaxa Nucleofector and Cell Line Nucleofector Kit V (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's protocol. For antibiotic selection 10 μg/ml of Blasticidin (Invitrogen) were used.

Western Blotting

Cells were washed twice with ice cold phosphate-buffered saline (PBS) and lysed in RIPA buffer containing phosphatase and protease inhibitor cocktails (ThermoFisher Scientific). Twenty μg of proteins from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Proteins were detected using antibodies against EpoR (goat polyclonal, 1:500 dilution; R&D Systems). As a loading control, horseradish peroxidase-conjugated polyclonal antibodies to β-actin (1:2000) and GAPDH (1:4000) (both from Santa Cruz) were used. Membranes were incubated with the primary antibodies overnight at 4°C; horseradish peroxidase-conjugated bovine anti-rabbit, anti-goat, or anti-mouse pre-absorbed antibodies for secondary staining were purchased from Santa Cruz and used in 1:5000 dilution. Immunoreactive bands were visualized using chemiluminescence (ECL Advanced Western blotting detection system; ThermoFisher Scientific).

Flow Cytometry

Cell surface EpoR expression was assessed by flow cytometry using phycoerythrin-conjugated mouse monoclonal EpoR antibody (FAB307P) and matching isotype-negative control (IC003P) (both from R&D Systems). Cell staining was performed according to the manufacturer's

protocol and measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Results were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR).

Immunoprecipitation and silver staining

EpoR specific immune precipitation (IP) was carried out from $1x10^8$ MDA-MB 436 cells after proteosome inhibition treatment to prevent the degradation of the protein. Cells were lysated in 1ml low stringency lysis buffer (PBS with 0.3% Igepal 640) and sonicated for 10s on ice. After the centrifugation step (at 10.000g for 10 min, 4C) 400ul of the supernatant with 2 ul of 1ug/ul Rabbit IgG and 10ul Sigma Protein-G agarose (50% beads) was used for IP. The mix was incubated for 30min in a rotator mixer at 4C. After centrifugation (at 3000rpm (approx.: 1000g) for 30sec at 4C) the supernatant was transferred to a new tube and 10ul (2ug) EpoR C20 rabbit polyconal AB was added to the supernatant and incubated for 2h at 4C in rotator mixer. 20ul of the Sigma Protein-G agarose (50% beads) was added to the IP mix and incubated overnight in rotator incubator at 4C. The beads were washed twice with PBS with 0.1% Igepal 640 and twice with PBS, the final volume of the samples was 40 ul. After running the Wester Blott the Pierce™ Silver Stain Kit was applied according to the manufactural's protocol (Thermofisher Scientific).

Results:

Variant EpoR forms expressed in human breast cancers

In the first stage of the project a cohort of clinically well characterized breast cancers with at least five years of clinical follow-up was selected and analyzed for EpoR expression using TaqMan assays specific for EpoR exon 8 and EpoR exon 3. Table 2. shows the major clinicopathological parameters of the tumor samples for the analysis. Out of the 189 selected samples the RNA isolation and the QRT-PCR reaction were successful in 157 cases.

Table 2. Major characteristics of the selected primary breast cancer samples

Type:	
Ductal	189
Subtype:	
Invasive micropapillary carcinoma (MP)	3
Invasive carcinoma of no special type (SNT)	149
Focal MP	36
Atypical medullary carcinoma	1
Grade:	
1	3
2	89
3	97
Side:	
Left	79
Right	106
Bilateral	4
Menopausal status:	
Postmenopausal	98
Premenopausal	50
Perimenopausal	4
S/P oophorectomy	27

Unknown	10
Lymphatic invasion:	
No	94
Yes	95
Metastasis	
Absent	118
Present	70
Unknow	1
Estrogen-receptor status:	
Negative	56
Positive	133
Progesterone-receptor status:	
Negative	83
Positive	104
Unknown	2
HER2 expression:	
0	74
1+	47
2+	26
3+	29
Unknown	13

Using quantitative RT-PCR out of the 157 samples 95 (61%) showed more than 2-fold change between the expression levels of the C and N-terminal regions of EpoR. By analyzing the correlation of the expression levels differences with clinicopathologic features, significant correlation was found between the expression differences of N and C terminal regions of EpoR and the lymphatic invasion of the tumors ($P<0.05$, Chi-Square test, Figure 1.)

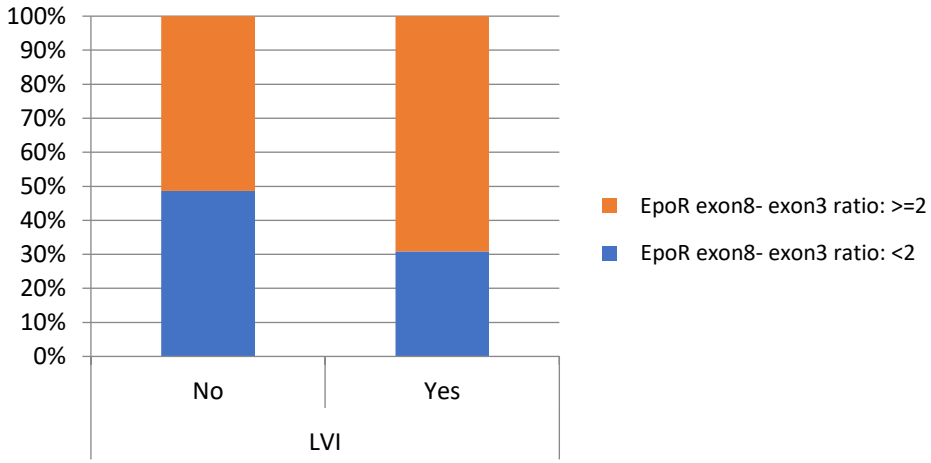


Figure 1. Expression differences between the EpoR exon 8- exon 3 and the lymphatic invasion of the tumor samples

Logistic regression model demonstrated the association of metastatic capacity and expression differences of extra and intracellular regions of EpoR in primary breast cancers ($P<0.05$, greater than 2-fold change).

The QRT-PCR results were presented on the XI. Public Health Conference (Szeged, 2017. 08.03-09.01) The title of the presentation was: The effects of erythropoiesis stimulating agents on the metastasis formation and survival in patients with breast cancer (Nagy Brigitta, Kiss István, Rákosy Zsuzsa).

Previously we showed that expression differences of N and C terminal regions of EpoR associated with the lymphatic invasion of the tumors. To explore this phenomenon further, we examined the expression profile of genes which play significant role in the lymphangiogenes and angiogenesis in the selected breast cancer samples. Based on the expression difference of the N and C terminal regions of EpoR, we selected 17 samples not showing expression differences, and 17 samples showing the highest level of difference and subjected for the TaqMan™ Array Human Angiogenesis assay analysis. The analysis revealed that the high level of expression difference between the N and C terminal regions of EPOR was associated with the elevated expression of two members of the Transforming Growth Factor beta signaling network (TGFA, TGFB) and one member of the Ephrin pathway (EPHB2). ($P<0.05$, greater than 2-fold change).

In order to further investigate the observed gene expression differences between the two regions of the receptor we applied SMART RACE PCR assay, which allowed us to examine the complete sequence of EpoR transcript in the case of ten tissue samples which showed the

highest expression differences between the two parts of the receptor. The results of the SMART RACE PCR assay visualized by gele-electrophoresis showed several distinct bands at ~1300 kb-800 bp including a common ~900 bp length sequence in most of the samples (Fig. 2 B). Sequence analysis presented that the most common cDNA isolated from this band started with a 14 bp intronic sequence and continued with only exons 5-8 of EpoR coding of the intracellular domain of receptor (Fig. 2 C). The exons 1-4 coding of the extracellular ligand binding domain of the receptor were completely missing. Our results indicate that variant forms of EpoR are present not only in established breast cancer cell lines, but in human clinical breast tumor samples as well (Fig.2 A-C).

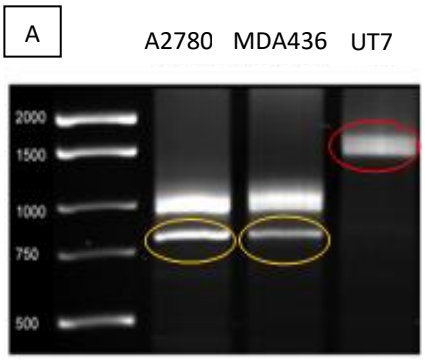
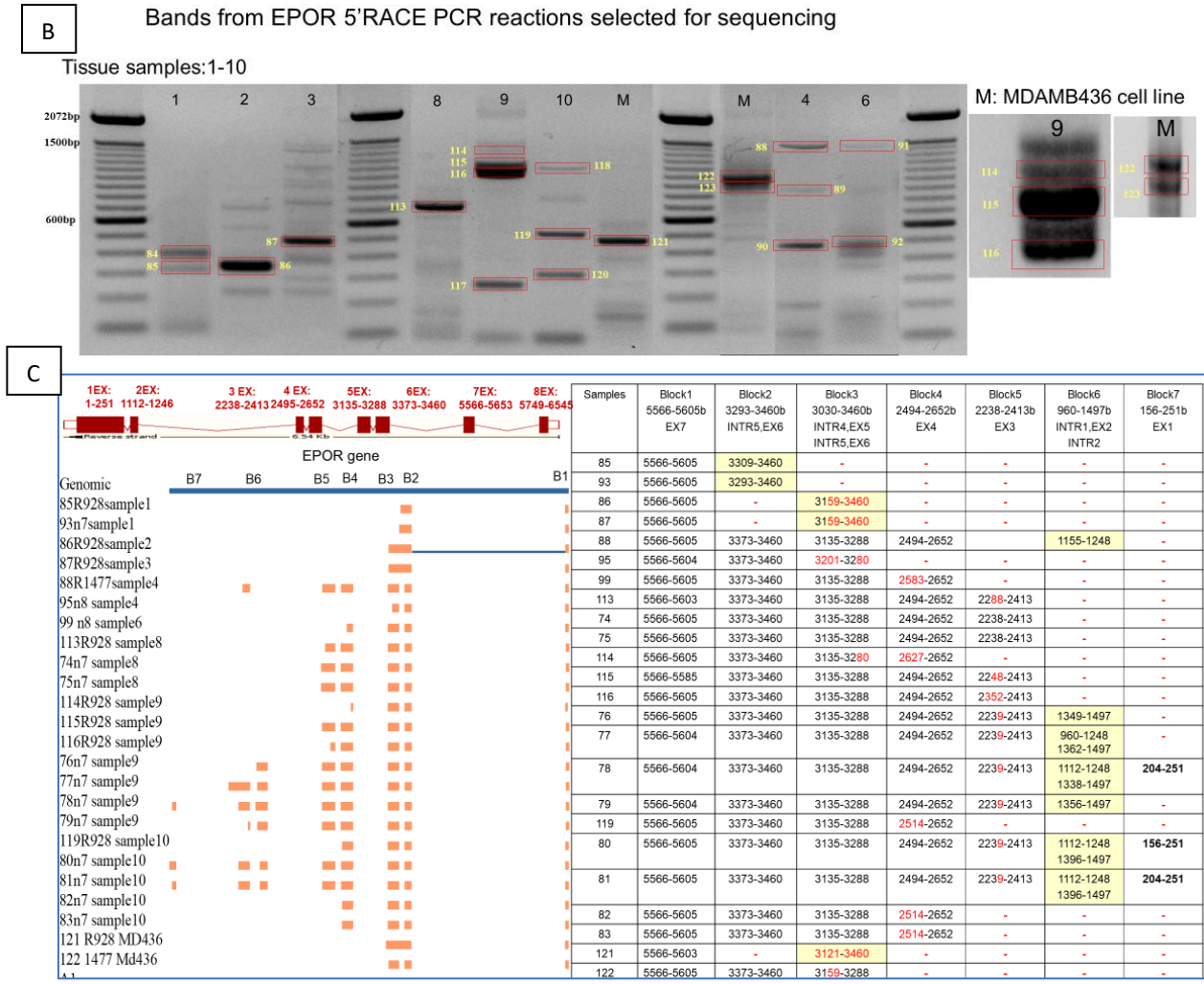


Figure 2.

A. *SMART RACE PCR system identify complete sequences of EpoR mRNA variants in A2780 ovarian and MDA- MB 436 breast cancer cells, in contrast to the full-length form present in UT-7 cells.*

Full length EPOR: ⬭ *, Isotype EPOR:* ⬭



Samples	Block1 5566-5605b EX7	Block2 3293-3460b INTR5,EX6	Block3 3030-3460b INTR4,EX5 INTR5,EX6	Block4 2494-2652b EX4	Block5 2238-2413b EX3	Block6 960-1497b INTR1,EX2 INTR2	Block7 156-251b EX1
85	5566-5605	3309-3460	-	-	-	-	-
93	5566-5605	3293-3460	-	-	-	-	-
86	5566-5605	-	3159-3460	-	-	-	-
87	5566-5605	-	3159-3460	-	-	-	-
88	5566-5605	3373-3460	3135-3288	2494-2652		1155-1248	-
95	5566-5604	3373-3460	3201-3280	-	-	-	-
99	5566-5605	3373-3460	3135-3288	2583-2652	-	-	-
113	5566-5603	3373-3460	3135-3288	2494-2652	2288-2413	-	-
74	5566-5605	3373-3460	3135-3288	2494-2652	2238-2413	-	-
75	5566-5605	3373-3460	3135-3288	2494-2652	2238-2413	-	-
114	5566-5605	3373-3460	3135-3280	2627-2652	-	-	-
115	5566-5585	3373-3460	3135-3288	2494-2652	2248-2413	-	-
116	5566-5605	3373-3460	3135-3288	2494-2652	2352-2413	-	-
76	5566-5605	3373-3460	3135-3288	2494-2652	2239-2413	1349-1497	-
77	5566-5604	3373-3460	3135-3288	2494-2652	2239-2413	960-1248 1362-1497	-
78	5566-5604	3373-3460	3135-3288	2494-2652	2239-2413	1112-1248 1338-1497	**204-251**
79	5566-5604	3373-3460	3135-3288	2494-2652	2239-2413	1356-1497	-
119	5566-5605	3373-3460	3135-3288	2514-2652	-	-	-
80	5566-5605	3373-3460	3135-3288	2494-2652	2239-2413	1112-1248 1396-1497	**156-251**
81	5566-5605	3373-3460	3135-3288	2494-2652	2239-2413	1112-1248 1396-1497	**204-251**
82	5566-5605	3373-3460	3135-3288	2514-2652	-	-	-
83	5566-5605	3373-3460	3135-3288	2514-2652	-	-	-
121	5566-5603	-	3121-3460	-	-	-	-
122	5566-5605	3373-3460	3159-3288	-	-	-	-

Figure 2 ***B:*** *Variants of EpoR mRNA forms were also detected in primary breast cancer samples. The marked bands were subjected for the further sequencing analysis.* ***C****: Left part of the figure demonstrates the results of the sequencing and in silico assembling, the right part of the panel shows the corresponding EpoR sequence in details. (Ex: exon, Intr.:intron)*

The most common EpoR Isotype mRNA sequence was the following:

ATTCCGCCCCCAGGTGGAGATCCTGGAGGGCCGCACCGAGTGTGTGCTGAGCAACCTGCGGGGCCGGACGCGCTA

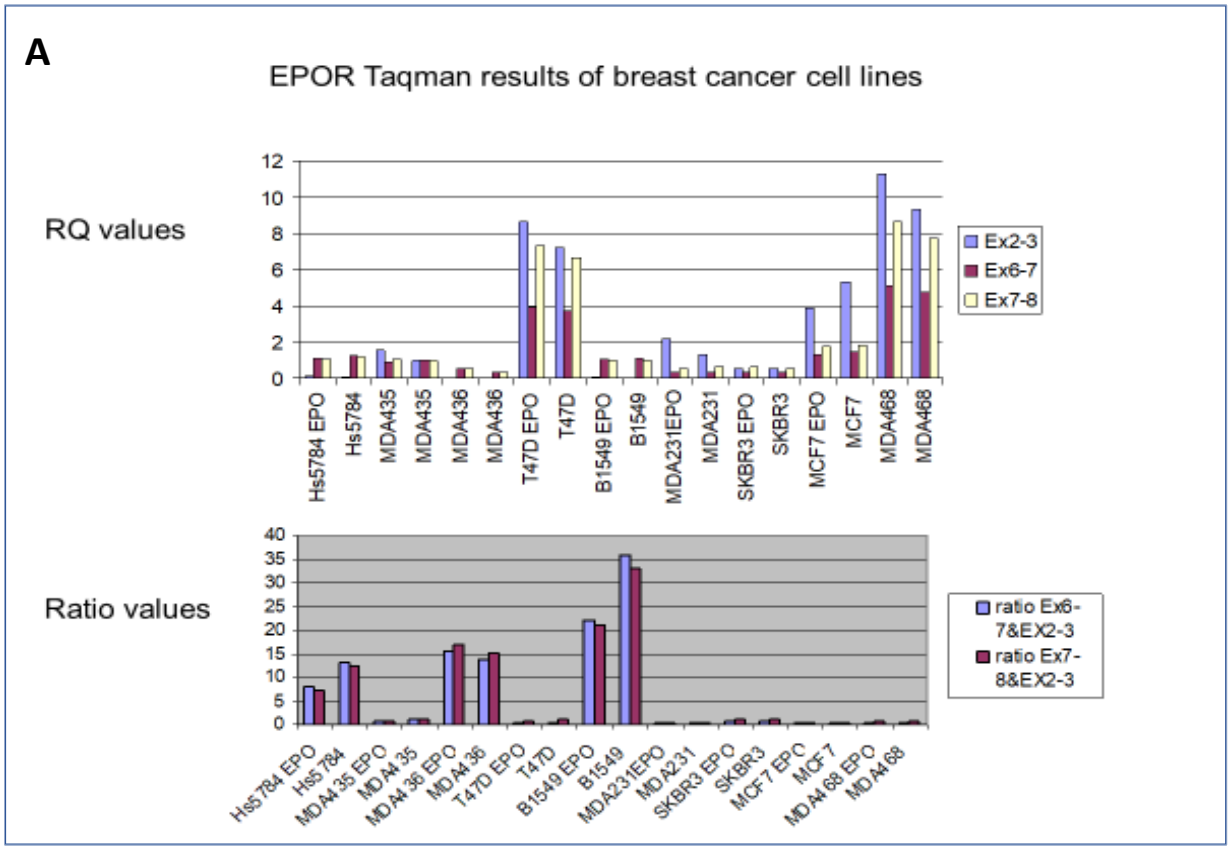
CACCTTCGCCGTCCGCGCGCGTATGGCTGAGCCGAGCTTCGGCGGCTTCTGGAGCGCCTGGTCGGAGCCTGTGTC

GCTGCTGACGCCTAGCGACCTGGACCCCCTCATCCTGACGCTCTCCCTCATCCTCGTGGTCATCCTGGTGCTGCT
GACCGTGCTCGCGCTGCTCTCCCACCGCCGGGCTCTGAAGCAGAAGATCTGGCCTGGCATCCCGAGCCCAGAGAG
CGAGTTTGAAGGCCTCTTCACCACCCACAAGGGTAACTTCCAGCTGTGGCTGTACCAGAATGATGGCTGCCTGTG
GTGGAGCCCCTGCACCCCCTTCACGGAGGACCCACCTGCTTCCCTGGAAGTCCTCTCAGAGCGCTGCTGGGGGAC
GATGCAGGCAGTGGAGCCGGGGACAGATGATGAGGGCCCCCTGCTGGAGCCAGTGGGCAGTGAGCATGCCCAGGA
TACCTATCTGGTGCTGGACAAATGGTTGCTGCCCCGGAACCCGCCCAGTGAGGACCTCCCAGGGCCTGGTGGCAG
TGTGGACATAGTGGCCATGGATGAAGGCTCAGAAGCATCCTCCTGCTCATCTGCTTTGGCCTCGAAGCCCAGCCC
AGAGGGAGCCTCTGCTGCCAGCTTTGAGTACACTATCCTGGACCCCAGCTCCCAGCTCTTGCGTCCATGGACACT
GTGCCCTGAGCTGCCCCCTACCCCACCCCACCTAAAGTACCTGTACCTTGTGGTATCTGACTCTGGCATCTCAAC
TGACTACAGCTCAGGGGACTCCCAGGGAGCCCAAGGGGGCTTATCCGATGGCCCCTACTCCAACCCTTATGAGAA
CAGCCTTATCCCAGCCGCTGAGCCTCTGCCCCCCAGCTATGTGGCTTGCTCTTAGGA

The results of the SMART RACE PCR were presented at the ESMO conference (MAP 2018, 2018 09. 14-15 Paris). *The title of the presentation was: Expression of erythropoietin receptor variant forms is associated with the lymphatic invasion and metastasis formation in breast cancer. The abstract was published as a supplement to the official ESMO journal Annals of Oncology. (Annals of Oncology, Volume 29, Issue suppl_6, 1 September 2018, mdy317.002, https://doi.org/10.1093/annonc/mdy317.002 Published: 16 September 2018).*

Beside of the EpoR mRNA expression profile, the EpoR protein expression was examined as well. EpoR protein specific experiments were not included in the original study protocol but taken into consideration that protein is responsible for the real function, and the relationship between mRNA and protein expression is not straightforward, it is important to examine that the tumor cells not only express the EpoR at mRNA level, but the translation takes place, too.

First, we assessed the expression of EpoR on the surface of breast cancer cell lines (MDA MB 231, MDA MB 436, MDA MB 468, SKBR3, TD47) by flow cytometry using a specific antibody detecting the N-terminal (extracellular) domain of EpoR. UT-7 Epo dependent erythroleukemia cells which known to express functional, wild type EpoR[32,33] was applied as a positive control. Although previously we could detect the expression of the exon 2-3 of EpoR mRNA by QRT-PCR in the cell lines with Epo treatment and without Epo treatment respectively (Fig.3A), no corresponding surface EpoR protein expression was seen by flow cytometry. In contrast the positive control UT7 cells showed marked surface EpoR expression (Fig. 3B).



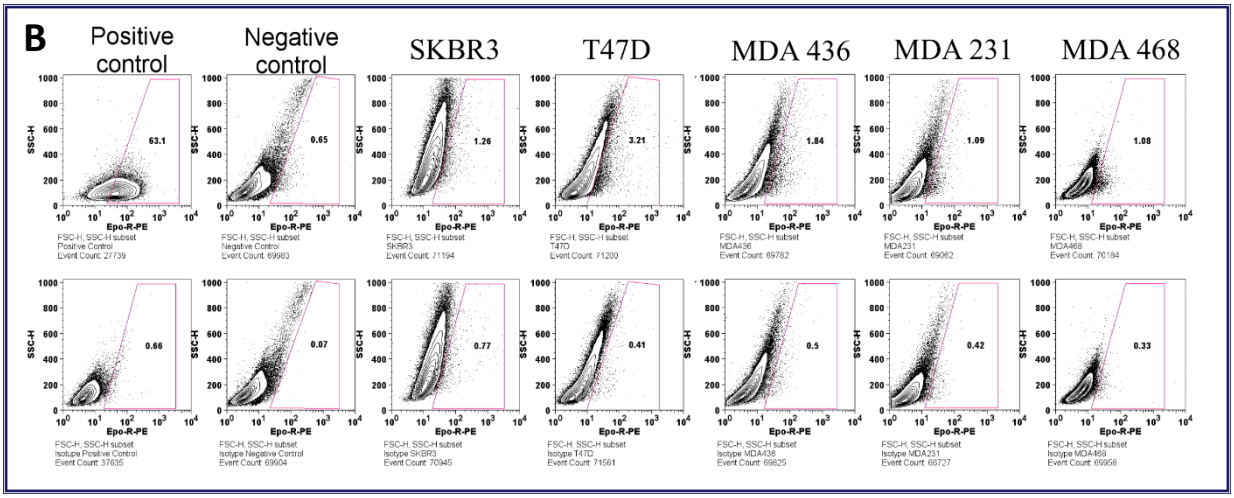


Figure 3. The expression of EpoR on the surface of breast cancer cell lines by flow cytometry using a specific EpoR antibody detecting the N-terminal (extracellular) domain of EpoR.

Antibody specific for the intracellular part of the EpoR was applied for the Western-blot experiments to detect the presence of the wild and the isotype forms of the receptor. Western-blot results also demonstrated no EpoR expression in the breast cells even if we could detect EpoR expression at mRNA level.

One explanation for the lack of protein detection could be that EpoR is expressed at levels below the threshold of detection of these assays. According to the literature data, very small number of EpoR for very short time is present in the erythroid cells (~1100 EpoRs per primary human EPC and ~300 per late stage erythrobast[34]. Therefore, EpoR specific "gigantic" immune precipitation (IP) was carried out from $1x10^8$ MDA-MB 436 cells after proteosome inhibition treatment to prevent the degradation of the protein. After the SDS page electrophoresis silver staining was applied, which is a subnanogram sensitivity method and it is ideal for visualization of low-level proteins. A distinct band was seen at ~66 kb in MDA-MB 436 cells which may indicate the EpoR protein (Fig. 4). This band was not seen in the negative control CHO cells. The distinct band was isolated; the sequence analysis of the protein is under investigation.

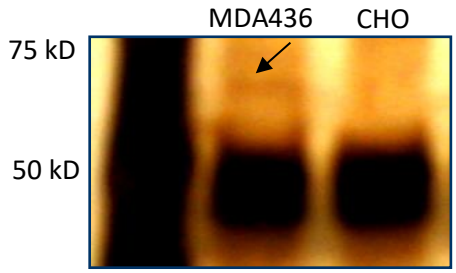


Figure 4. Result of the silver staining. Specific band can be seen in MDA-MB 436 cells at ~66 kD (narrow).

At this stage of the study we only had the opportunity to explore the protein expression on breast cancer cell lines. Using flow cytometry and Western-blot we demonstrated lack of EpoR protein expression in contrast with the mRNA expression data. Taken into consideration that the applied antibody-antigen based reactions for EpoR protein expression in the literature are very inconsistent, more sensitive, direct proteomic approach, like mass spectrometry, would be more appropriate approach to investigate further the presence of the EpoR protein in different cancer types.

Specific inhibition of the expression of variant EpoR forms in human breast cancer cell lines

In the next stages of the project we investigated the effect of the inhibition of full length and the variant EpoR form on the growth and sensitivity to Epo treatment of human breast cancer cells *in vitro* using RNA interference. Oligonucleotide cDNA inserts encoding short hairpin RNA (shRNA) specific for EpoR inserted into pSilencer 4.1-CMV hygro vector was generously provided by Dr. Acs. This was used in their previous study to inhibit the EpoR expression in A2780 ovarian cell line[31]. EpoR specific shRNA vector were transfected to MDA-MB 436 and SKBR3 cell lines using Amaxa nuclefector according to the protocol, but we failed to create stable cell clones. Eventhough we repeated the protocol several times with slight

modifications to optimize it, the cells could not survive the antibiotic selection, however the control cells transfected with vector without shRNA specific for EpoR could grow properly under the same conditions.

Meta-analysis of EPO effect on mortality and disease progression in patients with breast cancer receiving chemotherapy and ESA treatment

In parallel of the molecular biological experiments we started to collect the literature data for a meta-analysis of all currently available data from randomized controlled trials to evaluate mortality and disease progression in patients with breast cancer receiving chemotherapy and ESA treatment. This meta-analysis was not included in the original project, but the previous controversial results on the role of Epo/EpoR pathway in tumors, and the contradictory data of the ESA administration on the tumor progression and survival initiated us to further explore the effect of the ESA treatment within the clinical conditions.

There are two influential meta-analysis in the scientific literature about the effect of ESA treatment on survival and progression-free survival in cancer patients[35,36]. In a Cochran review in 2012 the authors included all cancers and different study types, experimental studies (clinical trials) and observational studies, as well[35]. They found that "There was strong evidence that ESAs increase mortality during active study period (hazard ratio (HR) 1.17; 95% CI 1.06 to 1.29, 70 trials, N = 15,935) and some evidence that ESAs decrease overall survival (HR 1.05; 95% CI 1.00 to 1.11, 78 trials, N = 19,003)." There was no statistically significant difference in tumor progression between patients receiving ESAs and controls. The ratio of the probabilities of complete response was 1.02 (95% CI: 0.98-1.06) comparing patients treated with ESAs to reference patients. "The risk ratio for thromboembolic complications was increased in patients receiving ESAs compared to controls (RR 1.52, 95% CI 1.34 to 1.74; 57 trials, N = 15,498)."

A recent meta-analysis by Aapro at al., included only patients with breast cancer and only clinical trials in their analysis which is justifiable, as patients in observational studies treated with chemotherapy and receiving or not receiving ESA treatment might have different distributions of prognostic factors[36]. This potential confounding by indication can be prevented by randomization in clinical trials. Aarpo at al. used odds ratios (OR) as effect measure in the publication. They found that "Deaths were reported for 571 of 2346 patients (24%) in the ESA groups and 523 of 2367 patients (22%) in the control groups [OR, 1.20; 95% confidence interval (CI) 1.03–1.40]. In seven studies reporting progression-related end points (N = 4197; ESA n =

2088; control n = 2109), the OR was 1.01 (95% CI 0.87–1.16) for ESA compared with control." Unfortunately, the use of odds ratio for the meta-analysis when the follow-up time is varying in the originally studies is questionable, as one cannot expect the odds ratio to be constant over time, thus pooling the results of studies with different length of follow-up is questionable. Instead, the hazard ratio is the appropriate association measure in this case. Therefore, our aim was to repeat the meta-analysis of Aapro at al. with the use of hazard ratio as the effect measure. Besides the methodological shortcoming of the meta-analysis of Aapro at al., another reason that called for the update of this analysis was the fact that a large clinical trial which included more patients than all the studies which were included in the meta-analysis of Aapro at al. have been published recently[37].

Methods:

We repeated the search applied by Aapro at al. on Ovid Medline Database restricted to publications published since 2014. We did not apply the original restriction regarding the type of publications. The search resulted in 37 hits. Among these, only one fulfilled the predefined inclusion criteria (clinical trial comparing patients treated with ESA or standard medical care suffering from anemia due to chemotherapeutic treatment of breast cancer)[37]. Thus, finally ten studies were included in our meta-analysis, nine which had already been included in the analysis of Aapro at al[36]., and the new clinical trial of Leyland at al[37].

When hazard ratios were not reported, they were estimated by dividing the estimated incidence rates in the ESA treated group and the control group. Confidence intervals of these ratios were estimated by exact Poisson regression. For the letter we used Stata/SE 15.0 statistical package. If no case occurred in any of the study group, one case was added in each group to make estimation of the confidence interval of the hazard ratio possible. This was necessary in the case of two trials[38,39].

We found some numerical inconsistencies in the meta-analysis of Aapro at al. Regarding O'Shaughnessy et.al 2005 study[39], in the Cochrane review and in the meta-analysis based on individual data[40], as well as the original paper reported one death in the ESA treated group and no death in the control group. However, in the meta-analysis of Aapro at al. they calculated with one death in the control group, as well. Furthermore, they calculated a study specific odds ratio of 2.94 which is not consistent with either the correct numbers or with the ones Aapro at al. reported.

Nitz et al. in 2014 reported safety data about 598 patients treated with darbapoetin alfa (DEA) and 601 controls[41]. Although the figures about the number of subjects were correctly reported in the meta-analysis of Aapro at al. regarding the number of patients randomized, no outcome data were reported about 64 patients who were not included in any analyses because of severe violation of inclusion criteria, thus the total numbers to calculate the odds ratio should have been reduced.

In the publication of Nitz at al. the hazard ratio of mortality was estimated for the first three years as 1.0, but the confidence interval of it was not reported[41]. We estimated the 95% confidence interval of the logarithm of the hazard ratio by normal approximation with a standard error of the logarithm of the hazard ratio calculated as

$$Se(lnHR)=\sqrt{(1/n\_1 +1/n\_0)}$$

Where lnHR is the logarithm of the hazard ratio, and n_1 and n_0 stand for the number of deaths in the study groups.

In the publication of Mobus at al. overall survival and relapse free survival were reported for a mean follow up of 5.17 years[42]. In a conference abstract ten-year survival and relapse free probabilities were also reported. However, hazard ratios were not reported. The effect of ESA treatment on survival and relapse free survival are more likely to be detected within a few years due to the acute nature of the treatment. Therefore, we used the data published in the original peer reviewed full text paper.

Regarding the publication by of Chang at al.[43], we used the published data on mortality within two years which were somewhat different from the figures used in the meta-analysis by Aapro at al. Risk of death after two years in each group was estimated as the number of deaths divided by the number of subjects allocated to the group. To estimate the hazards from the risks we used an exponential survival model. The standard error of the logarithm of the hazard ratio was calculated as described above. As no information was given about event free survival, this paper was not included in the analysis of this outcome. Contrary to this, it was included in Aapro at al. publication regarding progression free survival, but the source of the study specific figure used in that analysis is not known for us.

The publication of Prozanto at al[44] was included in the meta-analysis of progression free survival by Aarpo at al using data based on tumor assessment. However, in the paper of Prozanto at al. the figures about the number of patients with tumor progression an about the

number of deaths was given separately and not for the combined outcome of progression or death[44]. Therefore, we omitted this paper from the analysis of progression free survival.

To investigate the possibility of publication bias we studied the asymmetry of the funnel plot.

Results of the meta-analysis

There was no statistical evidence for large heterogeneity of the study specific effect neither regarding survival nor progression free survival. In case of survival, the p-value of the heterogeneity test was 0.79, and the I2 statistics was zero. In case of progression free survival, the p-value of the heterogeneity test was 0.49, and the I2 statistics was zero. However, we cannot assume that true effect of ESAs was the same in all studies included, as the group of included patients and the specific treatments applied were quite heterogenous. Therefore, we used standard random effect meta-analysis by DerSimonian and Laird using the statistical package of MetaXL which is an add-in to MS Excel[45].

Table 3. presents the major characteristics of the studies included in the analysis. It can be seen that the stage of cancer, and correspondingly the type of chemotherapy, as well as the specific ESA treatment were varying to a large extent.

Table 4. shows the study specific values of the time reference, the number of patients involved, and the number of the different outcomes together with the estimated study specific effect measures. The pooled estimate of the relative mortality comparing patients receiving and not receiving ESA treatment was 1,.09 (95% CI: 1.00-1.18) (Figure 6). A very large weight of 58% was attributed to the latest large study of Leyland[37]. The adverse effect of ESA treatment was somewhat less, only a non-significant 4% increase in the hazard of progression or death (HR: 1.04, 95% CI:0.96-1.12) compared to the control standard treatments (Figure 7). The effect of ESA was hardly influenced by the stage of disease and the type of chemotherapy. In case of studies which did not involve metastatic patients the hazard ratio of death was 1.10 (95% CI:0.87-1.39), and the hazard ratio of progression or death was 1.05 (95% CI: 0.83-1.35). The corresponding figures from the studies which involved metastatic patients were 1.12 (95% CI: 0.98-1.27) and 1.03 (95% CI:0.95-1.12).

Table 3. Major characteristics of the studies included in the meta-analysis

First author	Publication year	Stages included	Sample size		Cancer treatment stage	Active treatment
Adjuvant/neoadjuvant chemotherapy			**ESA**	**Control**		
Del Mastro[38]	1997	Stage II	31	31	Accelerated adjuvant chemotherapy	Epoetin α 150 U/kg 3 times weekly
O'Shaughnessy[39]	2005	Stage I-III	51	49	Adjuvant or neoadjuvant chemotherapy	Epoetin α 40 000 U once weekly
Nitz[41]	2014	Node positive non-metastatic	598	601	Adjuvant chemotherapy	Darbepoetin α 500 μg Q3W
Untch[46]	2011	Stage I-III	356	377	Neoadjuvant or adjuvant chemotherapy	Darbepoetin α 4.5 μg/kg body weight
Moebus[42]	2013	Stage II-IIIa	324	317	Adjuvant chemotherapy	Epoetin α 150 IU/kg 3 times
Mixed therapy						
Chang[43]	2005	25.8% in ESA, 19.6 in the control metastatic	177	177	Mixed adjuvant/neoadjuvant or metastatic disease	Epoetin α 40 000 U QW
Pronzato[44]	2010	47.7% Stage IV in ESA group and 44.0% in the control group	110	113	Mixed adjuvant/neoadjuvant or metastatic disease	Epoetin α 10 000 IU 3 times weekly
Metastatic disease						
Aapro et al.[47]	2008	Metastatic	231	232	Chemotherapy for metastatic disease	Epoetin β 30 000 U s.c. QW
Leyland-Jones[22]	2005	Metastatic	469	470	First-line chemotherapy for metastatic disease	Epoetin α 40 000 U once weekly or placebo
Leyland-Jones[37]	2016	Metastatic	1050	1048	Mixed adjuvant/neoadjuvant or metastatic disease	Epoetin alfa 40,000 IU subcutaneously once per week

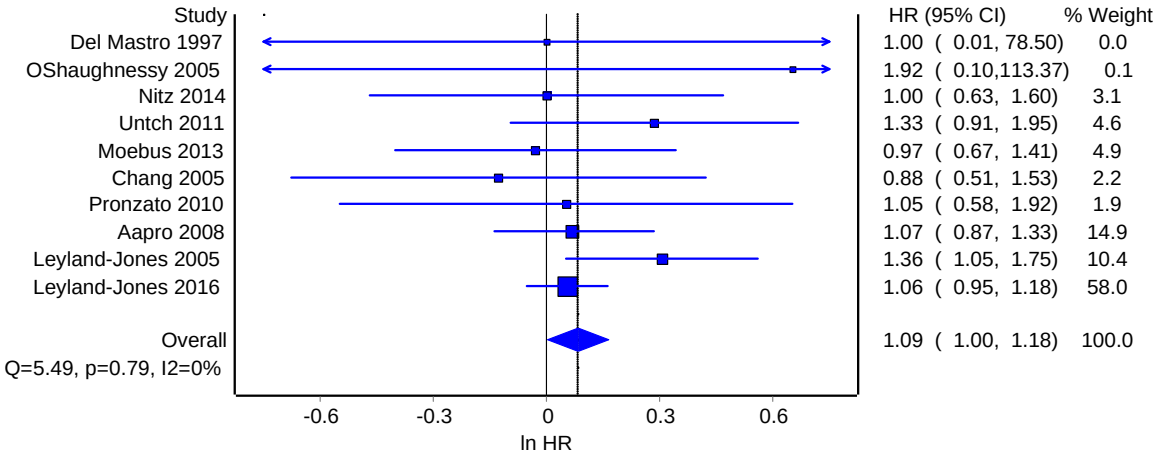
ESA: erythropoiesis stimulating agent, U: unit, s.c.: subcutanious, IU: international unit, Q3W: every 3 weeks, QW: once weekly

Table 4. Study specific numbers of outcomes and effect measures

First author	Publication year	Time reference	Number of deaths		HR (95% CI) mortality	HR (95% CI) progression or death	Relative frequency of thrombotic	
			ESA	Control			ESA	Control
Del Mastro[38]	1997	mean fup time: 0.27 years	0	0	1.00 (0.01-78.50)		0.0%	0.0%
O'Shaughnessy[39]	2005	at 0.5 year	1	0	1.92 (0.10-113.37)		2.0%	0.0%
Nitz[41]	2014	mean fup time: 3.25 years	33	37	1.00 (0.63-1.60)	0.85 (0.62-1.16)	4.0%	2.2%
Untch[46]	2011	mean fup time: 3.625	59	48	1.33 (0.91-1.95)	1.31 (0.99-1.74)	5.3%	4.5%
Moebus[42]	2013	mean fup time: 5.17	59	55	0.97 (0.67-1.41)	1.03 (0.77-1.37)	6.8%	3.2%
Chang[43]	2005	at two years	24	27	0.88 (0.51-1.53)		10.7%	7.9%
Pronzato[44]	2010	at 1 year	23	20	1.05 (0.58-1.92)		7.3%	6.2%
Aapro et al.[47]	2008	range: 2-3.58 years	169	169	1.07 (0.87-1.33)	1.07 (0.89-1.30)	12.6%	5.6%
Leyland-Jones[22]	2005	at 1 year	138	111	1.36 (1.05-1.75)	1.00 (0.82-1.22)	8.1%	7.0%
Leyland-Jones[37]	2016	6-18 month	681	656	1.06 (0.95-1.18)	1.03 (0.92-1.15)	3.3%	1.7%

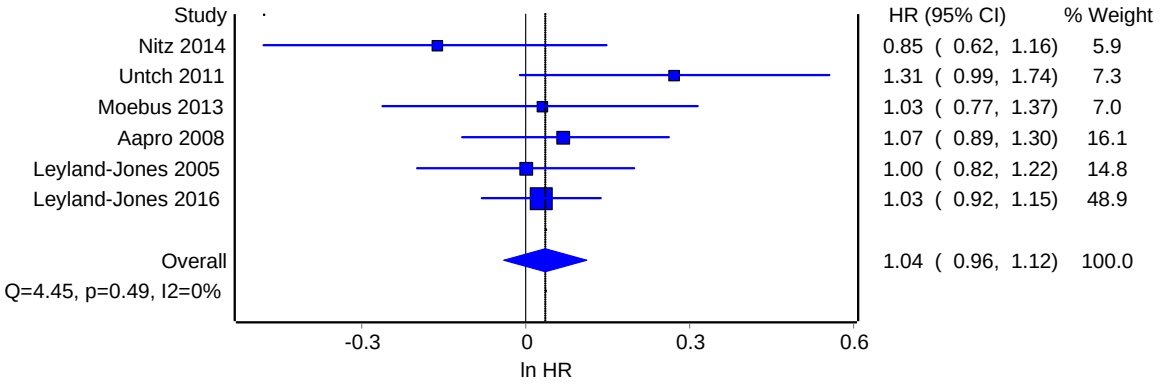
HR: hazard ratio, CI: confidence interval, Fup: follow-up

Figure 6. Forest plot of mortality.



HR: hazard ratio, ln HR: logarithm of the hazard ratio, CI: confidence interval

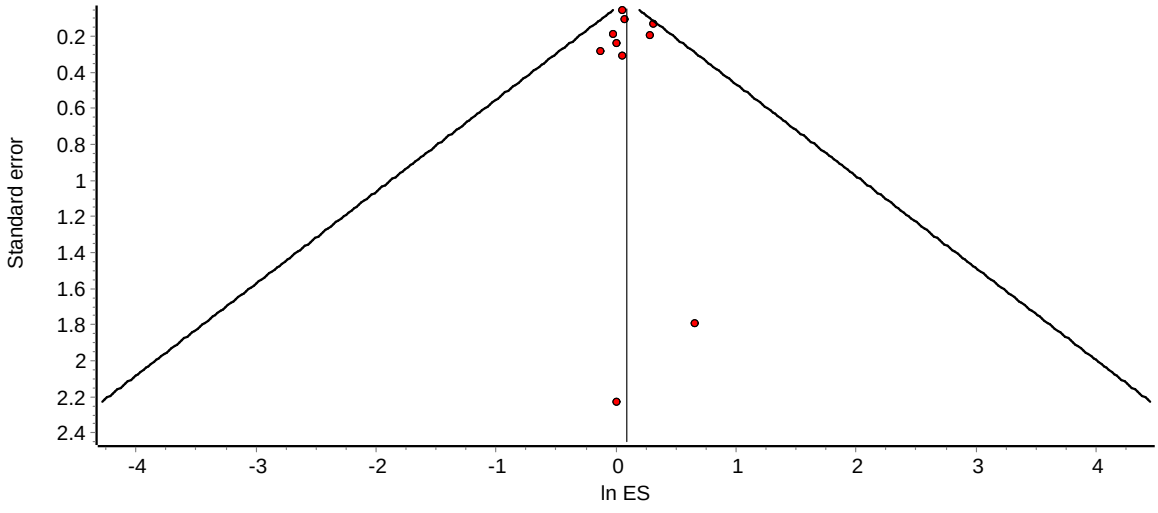
Figure 7. Forest plot of progression free survival (hazard of progression or death)



HR: hazard ratio, ln HR: logarithm of the hazard ratio, CI: confidence interval

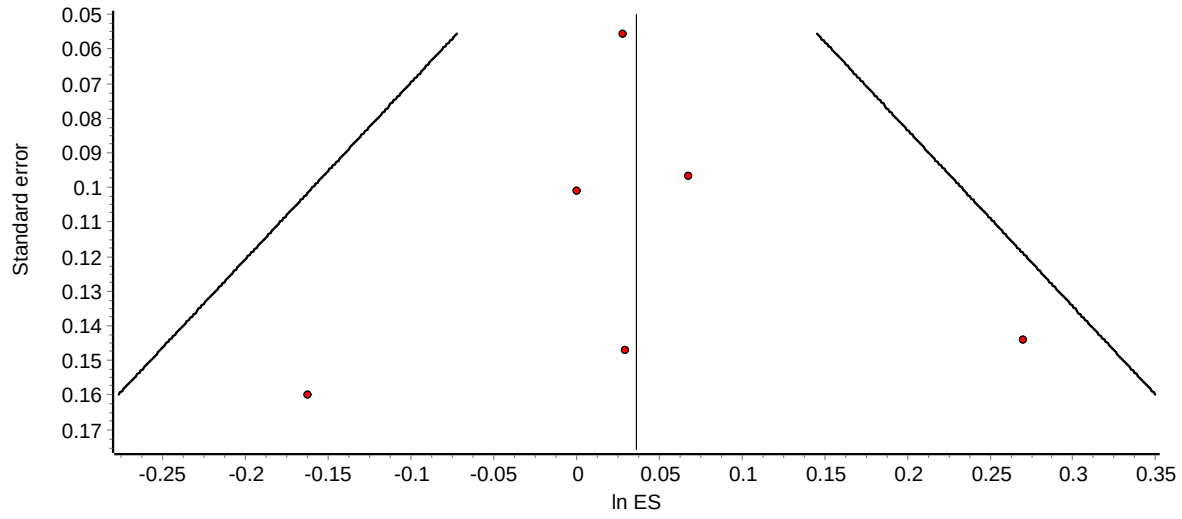
The funnel plot did not show asymmetry of the trial specific estimates in any region in either analyses not even in case of small studies. However, because of the small number of studies involved, we cannot exclude the possibility of publication bias, nevertheless the available evidence did not point into that direction (Figure 8 and 9.)

Figure 8. Funnel plot corresponding to mortality data



ln ES: logarithm of the effect size (hazard ratio)

Figure 9. Funnel plot corresponding to progression free survival data



ln ES: logarithm of the effect size (hazard ratio)

Discussion

Erythropoietin and its receptor are essential for erythropoiesis. EPO also has been reported to have effect on survival, proliferation of wide range of nonhematopoietic tissues and influences the progression of certain cancer types. It also has been shown that not only wild type of EpoR, but truncated receptor forms harbored too in different tissue types and malignancies[48-50]. With the goal of better understanding of the role of EpoR in the tumor biology our aim was to investigate the presence and the role of EpoR variants in the primary breast cancer samples.

The major results of the study are the following:

- QRT-PCR results demonstrated that EpoR mRNA is present in primary breast cancer samples.
- We also demonstrated that mRNA expression differences exist between the N and C terminal part of the EpoR in the breast cancer samples. The extracellular part of the receptor expressed in lower level than the its intracellular domain. The two-fold expression difference between the intra and extracellular part of the EpoR correlated with lymphatic invasion and the metastatic capacity of the tumors.
- Our results suggest that high difference between the N and C terminal regions of EpoR expression – lower expression of extracellular region of the receptor than the intracellular part – in breast cancer is associated with an elevated gene expression level of some members of the lymphangiogenesis and angiogenesis pathways (EPHB2,

TGFA, TGFB) compared to tumors with not showing expression differences between the two regions of EpoR.

- Smart Race PCR followed by sequence analysis showed that isoform of the EpoR with lack of the extracellular part coding exons is present in primary breast cancer samples.
- Although mRNA expression of EpoR was demonstrated in primary breast cancer samples, the ligand biding extra-cellular domain of EpoR protein and the intracellular part of the protein were not detectable using the commercially available antibodies either by Wester-blot or by flow cytometry, respectively.

The human EpoR gene is over 6 kb and contains 8 exons encoding a 508 amino acid, 66–105 kDa protein[51], which is a member of the type I cytokine receptor superfamily[52]. Not only the wild type of the receptor exist in cancer cells but also several EpoR splice variants[48-50]. With the concordance of findings of Arcasoy at al.[48] our results also demonstrated that EpoR isoforms exist in breast cancer. The isoform we characterized in the primary breast cancer samples had the lack of extracellular portion of the receptor. The high difference between the N and C terminal regions of EpoR expression in the samples indicating the presence of isoform of EpoR, was accompanying with lymphatic invasion and the metastatic capacity of the tumors. Furthermore, this was associated with elevated gene expression of TGFA, TGFB and EPHB2. TGFA and TGFB genes are involved in diverse set of cellular processes, including cell proliferation, recognition, differentiation, apoptosis. TGFB signaling plays a critical role in the breast cancer vascularization and metastasis formation via MAPK-MMP-9 pathway[53,54]. EPHB2 has a significant role in the angiogenesis, cell proliferation and migration. EPHB2 also binds to EPHB4 which activate the EPHB4 signaling and serves as a key modulator of vascular development and promoting angiogenesis[55,56]. Furthermore, EPHB4 can also form a heterodimer with EpoR and could serve as an alternative receptor for Epo[57]. According to the literature data, it might be worth further exploring the role of EpoR isoform in the promotion of the lymphangiogenesis and tumor progression with the involvement of EPHB2- EPHB4 axis, as well.

The EpoR protein expression data in the literature are very inconsistent, therefore we extended our examinations towards this direction as well. Previously it has been shown that various cancer cells, including cancers of the breast, ovary, endometrium, cervix, head and neck, prostate, kidney and lung, and melanoma, express EpoR at protein levels and display functional EpoR signaling[16-19,24-26]. However, many studies employing Western blotting and IHC often used commercially available polyclonal EpoR antibodies that have been shown to lack EpoR

specificity[58,59]. Patterson et al. recently investigated the expression of EpoR protein, the utilization of the erythropoietin receptor pathway and the response of tumor cells to human recombinant erythropoetin on disaggregated tumor cells obtained from 186 patients with colorectal, breast, lung, ovarian, head and neck, and other tumors. They could not detect any EpoR protein expression in tumor cells neither by flow cytometry nor by Western blot using antibody specific to the extracellular domain of the receptor. Furthermore, no functional response to rHuEpo was seen in freshly-derived primary tumor cell populations[60].
In our case we could detect the mRNA expression of the EpoR and their isoforms in the breast cancer samples, but we could not detect the EpoR protein expression. Nevertheless, the low-level EpoR cell surface expression in the erythroid and different cancer types emphasizes a need for cautious interpretation of apparent EPOR levels, and highlight the use of high-specificity reagents[61,62].

- We targeted the extracellular part of the receptor with specific shRNA and we failed to create stable cell clones.

The very recent study of Chan at al. could serve as an explanation for the observed phenomena. They showed that EPOR-depleted MDA-MB-231 and MDA-MB-435 breast cancer cells significantly reduced viable growth and colony formation compared to cells infected with scrambled shRNA by induced apoptosis through Bim. Their results indicate that the EPO-EPOR axis plays an important role in sustaining growth in these cell lines[63].

- We found statistically significant evidence that ESA treatment increased mortality by 9% in patients receiving ESA treatment for chemotherapy induced anaemia. The effect on progression or death was less, a statistically non-significant 4% increase of the hazard.

Our results are consistent with the findings of the referred Cochrane review and the meta-analysis of Aarpo et al.[35,36]. The newly published large trial by Leyland-Jones et al. did not change the direction of the already existing clinical evidence[37]. The Cochrane review and the meta-analysis of Aarpo et al. both found and increased risk of mortality with ESA treatment and no evidence for increased risk of tumor progression. If ESA increased the risk of death via increasing the progression of the tumors, one would not expect much less effect size regarding progression free survival compared to survival. But it was the case in our analysis. Similarly, the Cochrane review did not find evidence for difference in complete response, neither the meta-analysis of Aarpo et al. in progression-related end points in patients with or without ESA

treatment. If this is indeed the case, then other mechanism should explain the increased mortality. Available evidence suggests that ESA treatment may increase the risk of thrombotic events. The Cochrane review found that the risk of thromboembolic complications had been increased by 52% in patients receiving ESA treatment. As it can be seen in Table 4, the relative frequency of the investigated thrombotic events was all larger in all but one study included in our meta-analysis.

The manuscript to present results of the study is under preparation.

In summary, we can conclude, that molecular biological findings are still contradictory about the role of EpoR and EpoR isoforms in the tumor biology. Because of the controversial antibody-antigen based protein expression results, there is an urge to use more sensitive and specific direct proteomic methods to detect the EpoR protein for the better characterization of the possible effect of Epo treatment on the tumor progression via EpoR. Further studies needed to explore the possible interaction between the EpoR isoform and the Ephrin pathway and their role in the lymphatic invasion and the metastatic capacity of the breast cancer. Furthermore, according to the results of the clinical trials, the most likely interpretation of the current evidence regarding the risk of ESA treatment in breast cancer patients with chemotherapy induced anemia is that ESA increases mortality with approximately 10%, and its likely mechanism is that it increases the risk of thrombotic events. This should be carefully considered in the clinical practice when harms and benefits of the ESA treatment is considered for individual patients.

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