Endogenous myoglobin in human breast cancer is a hallmark of luminal cancer phenotype

Glen Kristiansen[1], Michael Rose[3], Cordelia Geisler[3], Florian R. Fritzsche[1], Josefine Gerhardt[1], Claudia Lüke[4], Axel-M. Ladhoff[4], Ruth Knüchel[3], Manfred Dietel[4], Holger Moch[1], Zsuzsanna Varga[1], Jean-P. Theurillat[1], Thomas A. Gorr[2]*, Edgar Dahl*[3]

[1] Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland
[2] Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland
[3] Institute of Pathology, University Hospital of the RWTH, Aachen, Germany
[4] Institute of Pathology, Charité - Universitätsmedizin Berlin, Berlin, Germany

* Co-senior authors

Presented in part at the XV International Conference on Dioxygen Binding and Sensing Proteins Meeting in Aarhus, Denmark, 2008

Short title: Myoglobin Expression in Human Breast Cancer

Word count: 4166

Character count (including spaces): 27869

Abstract word count: 248

Corresponding author:

Prof. Dr. Glen Kristiansen, M.D.
Institute of Surgical Pathology
UniversitätsSpital Zürich
Schmelzbergstr. 12
8091 Zürich
Switzerland
Email: glen.kristiansen@usz.ch
Tel +41 44 2553457
Fax +41 44 2554416

Abstract

Background: We aimed to clarify the incidence and the clinico-pathological value of non-muscle myoglobin (Mb) in a large cohort of non-invasive and invasive breast cancer cases.

Methods: Matched pairs of breast tissues from ten patients plus 17 breast cell lines were screened by qPCR for Mb mRNA. Additionally, 917 invasive and 155 non-invasive breast cancer cases were analysed by immunohistochemistry for Mb expression and correlated to clinico-pathological parameters and basal molecular characteristics including ERα/PR/HER2, FASN, HIF-1α, HIF-2α, GLUT1 and CAIX. The spatial relationship of Mb and ERα or FASN was followed up by double immunofluorescence. Finally, the effects of estradiol treatment and FASN inhibition on Mb expression in breast cancer cells were analysed.

Results: Mb mRNA was found in a subset of breast cancer cell lines, in microdissected tumors Mb was markedly upregulated. 71% of tumors displayed Mb expression in significant correlation with a positive hormone receptor status and better prognosis. *In silico* data mining confirmed higher Mb levels in luminal type breast cancer. Mb was also correlated to FASN, HIF-2α and CAIX, but not to HIF-1α or GLUT1, suggesting hypoxia to participate in its regulation. Double-immunofluorescence demonstrated a cellular co-expression of ERα or FASN and Mb. Also, Mb levels were modulated upon estradiol treatment and FASN inhibition in a cell model.

Conclusion: We conclude that in breast cancer Mb is co-expressed with ERα and co-regulated by estrogen signaling and can be considered a hallmark of luminal breast cancer phenotype. This and its possible new role in fatty acid metabolism may have fundamental implications for our understanding of Mb in solid tumors.

1. Introduction

Human myoglobin (Mb) is considered one of the best characterized proteins with more than 11,200 PubMed-listed publications since Kendrew *et al.* have presented the first three dimensional model of this molecule in 1958 (Kendrew, 1958). It is commonly described as a cytoplasmic hemoprotein that is solely occurring at milli-to micromolar concentrations in cardiac myocytes and type I and IIa skeletal muscle fibers of mammals. In myocytes, Mb is widely accepted to function as temporary “store” for oxygen, able to buffer short phases of exercise-induced increases in O_2 flux during which it supplies the gas to mitochondria (Ordway & Garry, 2004). Another, more controversially discussed role is Mbs´ facilitation of oxygen diffusion within muscle cells (Jürgens *et al*, 1994; Wittenberg, 1970). Although Mb knockout mice exhibited normal exercise capacity and no signs of compromised cardiac energetics due to multiple systemic compensations (Garry *et al*, 1998; Gödecke *et al*, 1999), follow-up studies stressed the importance of functional Mb in maintaining nitric oxide (NO) homeostasis in muscle through either scavenging (Flögel *et al*, 2001) or producing the NO molecule (Hendgen-Cotta *et al*, 2008). That way, Mb might participate in tuning vasodilatory responsiveness and protecting the respiratory chain from NO inhibition (Brunori, 2001). Further possible functions of Mb in muscle include synthesis of peroxides (Khan *et al*, 1998), scavenging of reactive O_2 species (Flögel *et al*, 2004) and binding of fatty acids (Masuda *et al*, 2008).

In humans, Mb is synthesized at concentrations of ~200-300 μM in striated muscle and, albeit at much lower levels, in a variety of human tumors including medullomyoblastoma (Smith & Davidson, 1984), thymolipoma (Iseki *et al*, 1990), acute leukaemia (Ruck *et al*, 1995) and desmoplastic small round cell tumors (Zhang *et al*, 2003). Following an accidental observation of positive Mb staining in several human carcinomas in 2001, we have since then systematically examined Mb expression in human breast cancer. Very recently, Flonta *et al.* described Mb in solid tumors including 31 breast cancer cases, however, this small cohort size precluded further statistical analyses (Flonta *et al*, 2009). Now, we are presenting the first comprehensive analysis of Mb expression in a large and representative cohort of human breast tissues encompassing normal tissue (n=56), Ductal Carcinoma in Situ (DCIS, n=155), invasive breast cancer (n=917) and breast cancer recurrences (n=76), enabling a portrayal of the

associations between clinico-pathological parameters and the range of Mb synthesis seen in mammary carcinomas. We also shed some light on unusual functions of Mb in cancer by looking at the impact of steroids on the steady state level of the protein and by examining Mb´s involvement in fatty acid metabolism.

2. Material and methods

2.1 Clinical materials/Patients

The matched tumor/normal samples of invasive ductal breast carcinomas and corresponding normal breast epithelium (n=10) analyzed in this study, have recently been described (Veeck *et al*, 2006). For immunohistochemistry, our study included tissue micro arrays of normal tissue, intratumoral *ductal carcinoma in situ* (DCIS), invasive breast cancer and breast cancer recurrences of patients diagnosed at the Institute of Surgical Pathology (University Hospital, Zurich, Switzerland), as described (Theurillat *et al*, 2007). Tumor histology was determined according to the criteria of the World Health Organization (2003), staging the disease followed UICC guidelines (2002). Tumors were graded according to Bloom and Richardson, as modified by Elston and Ellis (Elston & Ellis, 1993). Clinico-pathological characteristics of the patients/tumors are given in Table 1. For statistical analysis, only cases with clinical follow-up data were considered. The median observation time for overall survival was 59 months for patients still alive at the time of analysis. Two-hundred-and-twenty-five patients (24%) died during follow-up. Data on adjuvant therapy was not available. Appropriate review board consent has been obtained to allow use of these materials for research purposes.

2.2 Cell lines

The human mammary epithelial cell line MCF12A as well as the breast cancerous cell lines BT20, BT474, Cal51, EFM19, HBL100, Hs578T, MDA-MB231, MDA-MB361, MDA-MB453, MDA-MB415, MDA-MB436, MDA-MB468, MCF7, SKBR3, T47-D and ZR75-1 were obtained from the ATCC (Rockville, MD, USA) and cultured under recommended conditions.

2.3 Quantitative real-time reverse transcription PCR

For qPCR experiments we used the ABI Prism 7500 Fast SDS (Applied Biosystems, Darmstadt, Germany) with the following primers: MB (111bp) 5´-GGCATCATGAGGCAGAGATT – 3´ and 5´- TCTGCAGAACCTGGATGATG – 3`, GAPDH (289bp) 5’-GAAGGTGAAGGTCGGAGTCA-3’ and 5’-

TGGACTCCACGACGTACTCA-3’, ß-Actin (185bp) 5’- GGACGACATGGAGAAAATC-3’ and 5’- ATAGCACAGCCTGGATAGC-3’.

2.3 Western blots

MDA-MB468 cells were harvested with a lysis buffer containing 0.1% NP-40, 400mM NaCl, 1mM EDTA (pH8.0), 10mM Tris-HCl (pH8.0) and protease inhibitors. Protein extracts were electrophoresed on a 15% SDS-PAGE gel. Monoclonal antibody mouse anti-myoglobin (clone Z001, Zymed Laboratories, USA) was used for immunoblotting (1:1000 dilution). For equal loading control, blots were stripped and re-probed by monoclonal antibody mouse anti-β-actin at a 1:5000 dilution (#A5441, Sigma-Aldrich, Basel, Switzerland).

2.4 Immunohistochemistry and immunofluorescence

Tissue sections were processed using automated immunohistochemistry platforms (BOND, Labvision; Benchmark, Ventana) using the following antibodies and dilutions: Mb, clone Z001, Zymed Laboratories, USA (1:300). FASN, clone 3F2-1F3, Abnova, Taipeh, Taiwan (1:2000). HIF-1α, clone mgc3, Abcam, Cambridge, UK (1:400). HIF-2α, rabbit polyclonal, Novus Biologicals, Littleton, USA (1:150). GLUT1, rabbit polyclonal, Chemicon, Temecula, USA (1:1000). CAIX, rabbit polyclonal, Abcam, Cambridge, UK (1:300). Immunohistochemistry was evaluated by two clinical pathologists (GK, FFR). Intensity of Mb, GLUT1, CAIX and FASN was semiquantitatively scored as negative, weakly, moderately or strongly positive (0 to 3+). ERα/PR/HIFs were evaluated in percent of positive nuclei. Double Immunofluorescence (Mb/ER and Mb/FASN) was performed as described (Kristiansen *et al*, 2008).

2.5 Electron Microscopy (EM)

Tissues from two breast tumors were fixed with 2.5% glutaraldehyde, postfixed with osmium tetroxide, embedded in epoxy resin, cut with an ultramicrotome, mounted on 200 mesh copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM10 transmission electron microscope (Zeiss, Oberkochen, Germany) at 60 kV.

2.6 Expression of Mb gene after incubation of MCF-7 cells with ß-Estradiol

MCF7 cells ($8x10^5$) were seeded into a 10cm culture dish and 24h later 17-ß-estradiol (Sigma-Aldrich, Deisenheim, Germany) dissolved in 5 µl ethanol abs., was applied to the cells at a final concentration of 0 pM, 20 pM, 40 pM and 60 pM. Cells without 17-ß-estradiol and Ethanol served as internal control.

2.7 Expression of Mb gene after incubation of MDA-MB-468 cells with the FASN inhibitor C75

$3x10^5$ MDA-MB-468 cells were seeded into 6 well-plates and exposed to 10 µg/ml final concentration of the FASN inhibitor C75 (Sigma-Aldrich, St. Louis, MO, USA) for 8, 24 and 48h. RNA and proteins were isolated following standard procedures.

2.8 Statistical Analysis

Expression data were analyzed with the software package SPSS, version 16.0 (SPSS Inc., Chicago, USA). Fisher's exact and chi-square tests for trends were used to assess the statistical significance of associations between Mb expression and clinico-pathological parameters (Tab. 1). Univariate survival analysis was performed with univariate Cox analyses and Kaplan-Meier curves (Log rank test). Bivariate correlations according to Spearman were applied to the immuno-intensity of normal tissue, intraductal and invasive carcinomas.

3. Results

3.1 Human breast cancer tissue exerts a complex pattern of Mb expression

3.1.1 Mb is detectable on mRNA level in cell lines and human breast tumors

Following the initial observation of Mb immunoreactivity in conventional immuno-histochemistry analyses, Mb transcript levels were determined in breast biopsies and breast cell lines. Whereas low levels of Mb mRNA were detectable in four of ten cases of healthy breast tissue (Fig. 1A), Mb expression was upregulated in nine of ten matched normal / tumor tissue samples with a median tumor-to-normal up-regulation of 352 fold. With regard to breast cell lines, Mb mRNA was not detectable in benign MCF12A epithelial cells as well as in MDA-MB436, Hs578T, Cal51 tumor cells (Fig. 1B). Ten breast cancer cell lines (MDA-MB231 to MCF7, Fig. 1B) expressed detectable but low amounts of Mb mRNA while three breast cancer cell lines contained abundant quantities of the Mb transcript, i.e. EFM19, MDA-MB415 and MDA-MB468 cells. Regarding the protein abundance, we used a commercial chemiluminescence based Mb-assay and determined the amount of Mb protein present in normoxic 10^6 MDA-MB468 cells to correspond to ~65 ng or 4 pmol (assuming a mean cell size of 20 micrometer and sphere shaped cells).

3.1.2 Large scale immunohistochemical analysis of Mb protein in breast tissues

Using a validated monoclonal Mb antibody (Fig. S1), we next analysed Mb expression on tissue microarrays containing normal tissue (n=56), intratumoral ductal carcinoma in situ (DCIS; n=155), invasive breast cancer (n=917, clinico-pathological parameters are given in Table 1) and 76 recurrences of invasive tumors. In normal breast tissue, staining was observed in secretory luminal epithelial cells, but not in myoepithelial cells (Fig. 1C, arrowhead), with single secretory cells (bold arrow) staining considerably stronger than adjacent secretory cells (Fig. 1C, thin arrow). Altogether, in normal breast seven percent of cases were completely negative for Mb, 49% were weakly positive, 39% stained moderately positive and five percent of tissues stained strongly (Fig. S2). DCIS showed altogether a more abundant Mb staining (Fig. 1D): 12% of DCIS cases were negative, 38% weakly positive, 39% moderately positive and 11% were strongly positive. In invasive carcinoma, the number of Mb negative cases was markedly higher than in

normal tissue: 29% were negative, 32% were weakly positive, 30% were moderately positive (Fig. 1E) and 9% were strongly positive (Fig. 1F). A spotted, mosaic like expression pattern was frequently seen in DCIS and invasive carcinoma (Fig. 1E). In tumor recurrences, 30% cases were Mb negative, 22% weakly positive, 33% moderately positive and 15% stained strongly (Fig. S2). Apparently, although the mean Mb positivity is not significantly altered during malignant progression, an increasing polarization in Mb expression is noted in the increased number of negative and strongly positive cases from normal tissue to tumor recurrences.

3.2 Electron microscopy: Mb is found without striated muscle elements

Since myoglobin has been described as a marker of rhabdomyoid differentiation, we analyzed two breast tumors with strong Mb immunoreactivity by transmission electron microscopy (Fig. S3A/B). However, no striated muscle elements could be observed in these cases, suggesting that increased expression of Mb occurs independently of rhabdomyoid tumor differentiation.

3.3 Myoglobin expression in invasive breast cancer shows a complex correlative pattern to endogenous markers of Hypoxia (HIF-1α, HIF-2α, GLUT1 and CAIX)

A subset of invasive breast cancer cases (n=150) was additionally analyzed for protein level expression of the hypoxia inducible (transcription) factors 1α and 2α (HIF-1α, HIF-2α) which are generally considered the key regulators of the hypoxic response in cells and tissues, and their target genes glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX). Mb expression correlated significantly with HIF-2α and CAIX but failed to correlate with HIF-1α or GLUT1 (Table 2).

3.4 Tumor-derived myoglobin expression is linked to estrogen receptor status and better prognosis and may present a marker for luminal type breast cancer

In invasive breast carcinomas Mb expression was associated with better histological tumor differentiation according to BRE-grading (correlation coefficient (cc) =-0.116, p=0.001). Mb did not correlate with pT stage, nodal status or tumor type. Mb expression was positively correlated with estrogen (ERα) and progesterone receptor (PR) positivity (cc=0.206, p=0.001 and cc=0.180, p=0.001) and negatively with the

myoepithelial/basal phenotype marker CK5/6 (cc=-0.120, p=0.001). No significant correlation was found with Her2, Ki-67 fraction or CD34-microvessel density.

Towards the prognostic value of Mb and clinico-pathological parameters, a univariate Cox analysis confirmed histological tumor grade, pT stage, nodal status and hormone receptor (ERα/PR) status as significant predictors of overall survival in our patient cohort (Supplemental Table 1). High Mb expression was also significantly associated with longer overall patient survival (five-year survival rate of Mb-pos. cases 83% *vs.* 75% in Mb-negative cases, Fig. 2A) but lost significance in a multivariate Cox analysis which included pT, pN, BRE-grade, ERα and Mb (not shown). In a multivariate survival analysis restricted to ERα, PR (as markers of luminal phenotype) and Mb, only PR remained a significant prognosticator (HR=0.62, p=0.004), whereas Mb yielded a trend towards a prognostic value (HR =0.75, p=0.06) and ERα showed none at all (HR=0.74, p=0.11). However, in a Kaplan Meier analysis stratifying tumors in groups that were either a) negative for ERα and Mb, b) positive for ERα or Mb or c) positive for both ERα and Mb, it became apparent that Mb does add some prognostic information to the ER status (Fig. S4), since tumors that were either positive for Mb or ER showed an intermediate course in comparison to tumors that were negative or positive for both markers (p=0.001).

We additionally classified Mb levels in correlation with a publically available breast cancer expression dataset (Farmer *et al*, 2005) (GEO Profiles, http://www.ncbi.nlm.nih.gov/sites/entrez, GDS1329). Here, Mb levels are significantly higher (p=0.005, Kruskal-Wallis test) in the group of luminal tumors compared to basal or apocrine types. These findings along with the positive correlation of Mb with ERα, the better tumor differentiation with improved prognosis, and the negative correlation with the basal phenotype marker CK5/6 all point to Mb as a marker of luminal tumor differentiation. This association of Mb with characteristics of the luminal tumor subtype, namely ERα positivity led us to investigate this point further.

3.6 Mb expression is regulated by estrogen signaling in silico and in vitro and co-localizes with the estrogen receptor

Publically available DNA array expression data (Geoprofiles, GDS2324) (Coser *et al*, 2003) had already indicated a tight co-regulation of Mb and ERα mRNAs in the breast cancer cell line MCF7 by showing that estrogen starvation was able to induce, while estrogen application suppressed, Mb and ERα transcripts in either a time or dose dependent manner (Fig. S5; ERα = ESR1 in figure). We repeated this experiment and could confirm the *in silico* data also *in vitro* in that application of 17-β-estradiol to ERα positive MCF7 breast cancer cells repressed Mb expression dose dependently (Fig. 2B).

This co-silencing of ERα and Mb expression by estrogen application to a breast cancer cell line prompted us to analyze the spatial association of both proteins by double immunofluorescence. In normal lobular breast parenchyma, approximately 90% of the strongly Mb positive secretory cells interspersed in the secretory cell layer that we had already noted in the chromogenic immunohistochemistry, demonstrated a cellular co-localisation (Fig. 2E, left) of cytoplasmic Mb (Fig. 2C, left) and nuclear ERα (2D, left) staining. In invasive breast carcinomas, this co-expression still was apparent in well differentiated carcinomas (2E, right), whereas in moderately-poorly differentiated tumors this overlap was less prominent (not statistically evaluated).

3.7 Non-respiratory functions of non-muscle Mb?

The amount of Mb detected in human breast cancer tissue and MDA-MB468 cells was similar in Western blot analysis (data not shown) and equaled 65 ng per 10^6 tumor cells as described above. Since this minute amount of Mb is unlikely to play a significant role in the maintenance of cellular oxygenation, Mb functions alternative to respiration support have to be considered. Recently, Mb was described again as a fatty acid (FA) binding protein and suggested to have a role in the transport of FAs in oxygenated cells (Gloster & Harris, 1977; Masuda *et al*, 2008; Yackzan & Wingo, 1982). This prompted us to investigate the possible co-localization of Mb and fatty acid synthase (FASN) in both healthy and cancerous breast tissue. FASN catalyses the synthesis of unbranched fatty acids and is upregulated in the broad majority of malignant tumors (Menendez & Lupu, 2007). In a direct comparison of FASN expression with Mb in a subset (n=293) of our

breast cancer cohort, a highly significant correlation of both proteins was found (CC=0.297, p=0.001). Moreover, in normal breast tissues, again, a striking spatial concordance in the expression of Mb and FASN was seen (Fig. 3A-C, left), which was also partially retained in cancer (Fig. 3A-C, right).

To check for a possible functional association of Mb expression and intracellular fatty acid levels, we used the FASN inhibitor C75. This inhibitor has been characterized as FASN specific, leading to an almost immediate and irreversible enzyme inhibition (Kuhajda *et al*, 2000). In MDA-MB468 cells, we observed a strong time dependent down-regulation of Mb on transcript (Fig. 3D, left) and protein level (Fig. 3D, right) upon FASN inhibition in comparison to control cells, suggesting that non-muscle Mb expression is indeed regulated by intracellular fatty acid levels. Thus, Mb in breast carcinomas and cancer cells might be involved in controlling fatty acid metabolism.

4. Discussion

This report is the first systematic examination of myoglobin (Mb) expression in a large cohort of breast cancer specimens that allows clinical and molecular correlations and furthermore points to unexpected functional facets of this hemoprotein. We first noticed a supposedly aberrant Mb immunoreactivity in a small series of breast carcinomas. To address whether Mb is being actively produced by breast cancer cells or, rather taken up from e.g. adjacent musculature as suggested by Eusebi *et al.* more than 25 years ago (Eusebi *et al*, 1984), we analyzed its expression both *in vitro* and *in vivo*. On transcript level, we found Mb strongly upregulated in breast tumors in comparison with adjacent normal ductal tissue. Mb mRNA was also detectable in breast cancer cell lines, in a small subset even at surprisingly high levels. This underscored the active expression of endogenous Mb in ordinary invasive ductal breast cancer, which were neither muscle-invasive nor did they, as assessed by light microscopy, exhibited any rhabdomyogenous differentiation. Transmission electron microscopy did also not reveal any striated muscle elements in two strongly Mb positive breast cancer cases. We therefore conclude that Mb is *de novo* expressed in breast cancer cells although rare cases of so-called metaplastic carcinomas of the breast might exist where Mb-positivity stems from the rabdomyogenous differentiation of the cells (Jamieson & Rudland, 1990; Yang *et al*, 2003).

On the basis of these encouraging preliminary findings, a large recently described (Theurillat *et al*, 2007) cohort of primary breast cancer including 917 invasive carcinomas and 155 cases of Ductal Carcinoma in situ (DCIS) was analysed for Mb expression. Specificity of the Mb antibody was confirmed through the peptide blockage of the immunohistochemical signal (Fig. S1). In total, 40% of invasive carcinomas showed moderate to strong Mb expression and the rate is even slightly higher in DCIS. However, Mb was also consistently seen in mature secretory epithelia of the healthy breast showing a basal expression in most cells with a particularly pronounced expression in estrogen receptor alpha (ERα) positive cells, as we noted later. Contrary to earlier reports that found normal epithelia invariably Mb negative (Flonta *et al*, 2009), luminal cells in healthy breast clearly have the ability to express Mb at detectable levels. The recently published study of Flonta *et al.,* that came out during the preparation of this manuscript, identified endogenous Mb in various human cancer cell lines and human epithelial tumors including

breast cancer and elucidated several signals including nitric oxide, oxidative stress and mitogens (EGF and serum growth factors) but also hypoxia, that were able to stimulate Mb expression in cultivated MCF7 breast cancer cells (Flonta *et al*, 2009). However, their study, which encompassed only 31 breast cancer cases, lacked any prognostic or detailed clinico-pathological data.

Regarding its occurrence in breast cancer, we found that Mb is preferentially detected in better differentiated, hormone receptor positive tumors and is associated with a significantly better prognosis. All these features are characteristics of the so called luminal subtype of breast cancer according to Perou *et al.* (Perou *et al*, 2000) which shares many molecular similarities with normal secretory epithelia including a strong expression of cytokeratins typical of mature secretory epithelia (CK8/18), hormone receptor (ERα/PR) positivity and negativity for HER2 and the basal cell cytokeratins CK5/6. Further support for considering Mb a diagnostic marker of the luminal breast cancer subtype comes from the *in silico* analysis of Farmer *et al.'s data*, in which Mb transcript levels were highest in tumors classified as the luminal subtype (Farmer *et al*, 2005). Our immunofluorescent double stainings further detected co-localisation of cytoplasmic Mb and nuclear ERα in secretory cells of normal breast tissue. The same intracellular co-localization was found in strongly Mb and ERα-positive invasive ductal carcinomas. Beyond this spatial co-existence, Mb and ERα transcripts are also co-ordinately and dose-dependently down-regulated when MCF7 cells are subjected to an estrogenic (E2 = 17-β-estradiol) treatment protocol as demonstrated by *in silico* (Fig. S5) and *in vitro* (Fig. 3B) analyses. In this context it is of relevance to highlight the observation of Cheng *et al.,* who described the inverse relationship of E2 and ERα (which we found co-expressed with Mb) levels in normal breast epithelia during the mammalian menstrual cycle (Cheng *et al*, 2005). These authors further point out that elevated expression of ERα, occurring, for example, in postmenopausal women as a result of the loss of E2 signaling, is indicative for non-proliferating breast cells. The hitherto undescribed co-regulation of Mb with ERα by estrogen likely underlies the co-expression of ERα with Mb in differentiated luminal cells with their reduced proliferation, which, in turn, might explain the favorable prognosis of Mb positive tumors. The negative association of tumoral Mb expression and a pre-menopausal status further fits the interpretation of Mb as an

estrogen silenced gene, since premenopausal patients (high estrogen serum levels) have in comparison to postmenopausal patients (lower estrogen serum levels) tumors with lower Mb expression levels. In any instance, since the prognostic value of Mb is limited in comparison to ER or PR, we do not suggest its use as a primary marker to identify luminal phenotype carcinomas for therapy planning. However, Mb might gain clinical relevance if it could be validated as a novel therapy target of cancer, e.g. by applying carbon monoxide in sub-toxic concentrations, as has been suggested by Dr. Wittenberg at the O2BIP meeting in Aarhus in 2008, which clearly warrants further study. This is the more important, since luminal type breast cancer, although generally more favourable in course, does not respond well to conventional chemotherapy (Bhargava *et al*).

Mb has different known or alleged functions in muscle tissue including short-term O_2 storage and buffering, facilitating O_2 diffusion, scavenging of NO and ROS and also the reverse (peroxidase activity, NO production) and might be involved in fatty acid metabolism (Flögel *et al*, 2004; Flögel *et al*, 2001; Hendgen-Cotta *et al*, 2008; Khan *et al*, 1998; Masuda *et al*, 2008; Ordway & Garry, 2004; Wittenberg *et al*, 1975; Wittenberg, 1970). The functions of Mb in non-muscle tissues are elusive so far. More than 50 years after Thomlinson's and Gray's pioneering discovery to link radiosensitivity with oxygen tension in tumor tissue (Gray *et al*, 1953; Thomlinson & Gray, 1955), the existence of regional hypoxia in *most* solid tumors (for review: (Brown, 1999; Brown & Wilson, 2004; Thews *et al*, 1998; Vaupel *et al*, 1991) including breast cancer is of high clinical relevance (Vaupel *et al*, 1992). Using the exogenous hypoxia marker pimonidazole Arcasoy *et al.* concluded from a series of 26 breast cancer cases that 62% of tumors of the breast are pimonidazole-positive and thus hypoxic (Arcasoy *et al*, 2002). Since this rate of positivity is in the range of Mb positivity we observed in breast cancer, we had originally hypothesized to find an association of Myoglobin with tissue hypoxia. Also, the demonstration of hypoxia driven Mb induction in a cell line model by Flonta *et al.* fitted this interpretation very well (Flonta *et al*, 2009). However, our *in vivo* data, correlating Mb expression with acknowledged endogenous markers of hypoxia, revealed a rather more complex and heterogeneous picture. There was no correlation with HIF-1α and its target gene GLUT1 which could either mean that Mb is not significantly regulated by hypoxia *in vivo* or that HIF-1α is insufficiently present and active due to the amelioration of tissue hypoxia by expressed Mb. However, given the picomole concentrations of Mb protein we

have measured per million of the maximal Mb-producing MDA-MB468 cell line (Fig. 1B), the latter scenario is rather unlikely to explain this lack of correlation. On the other hand, there is a significant correlation of Mb to HIF-2α and the HIF-1/-2 target gene CAIX, which is in line of at least a partial control of Mb expression by hypoxia. Yet, expression of Mb in breast epithelia can clearly be regulated irrespective of oxygen levels (i.e. signals in normal secretory ductal cells). The fact that Mb in breast malignancies links with HIF-2α rather than HIF-1α results perhaps from the mutually exclusive status between active HIF-1α and ERα/PR signalling in breast cancer cells (Kurebayashi, 2003; Massaad-Massade, 2004). In this sense, it is the positive correlation between Mb and ERα/PR positivity which might preclude a significant linkage of Mb with HIF-1α. All this evidence only underscores the complex regulation of the Mb gene in breast carcinomas, where hypoxia/HIF signaling is just one of several stimuli to modulate the abundance of this hemoprotein.

To our knowledge, so far only two studies have investigated the functions of myoglobin in *in vivo* models using artificial Mb expression systems. Nitta *et al.* induced Mb expression in hepatocytes by an adenoviral gene transfer in rodents, with the effect, that these hepatocytes were significantly more hypoxia resistant (Nitta *et al*, 2003). Galluzzo *et al.* were the first to introduce Mb into tumor cell lines and engineered A549 human lung carcinoma cells to ectopically express mouse Mb (Galluzzo *et al*, 2009). Their experimental Mb expressing tumors displayed reduced or no hypoxia, minimal HIF-1α levels, decreased vessel density and finally a more differentiated cancer cell phenotype. Also, largely suppressed local and distal metastatic spreading was observed. The authors assume that these beneficial outcomes of Mb over-expressing tumors result primarily from the reduction of tumor hypoxia (Galluzzo *et al*, 2009). Although it is tempting to compare their mouse model *in vivo* findings with our *in vivo* observations from human patients, given that higher Mb levels correlate with less aggressive tumor behavior, both situations are quite different. We have estimated the amount of endogenous Mb in normoxic MDA-MB468 breast cancer cells to equate to ~65 ng or 4 pmol of Mb protein present in 10^6 cells. This quantity is certainly far below the high μM levels reached by the lentiviral gene transfer (Galluzzo *et al*, 2009). While such excessive amounts of ectopic Mb in tumor cells can with reason be assumed to have a significant

impact on tumor respiration and tumor growth, the endogenous picomole quantities of Mb we detected are unlikely to confer meaningful O_2 storage/buffering capacity to the cell, as already mentioned above. Thus, for these cells, functions of Mb that are not directly linked to the binding and transport of O_2 have to be considered to understand the physiological relevance of this protein in breast cancer.

One of these functions of Mb that might also be tremendously relevant for tumor cells is the control of fatty acid metabolism. In a multitude of tumors, growth is accompanied with increased fatty acid synthesis and consequently enzymes catalyzing these steps are up-regulated and can be used diagnostically and therapeutically (for recent reviews see (Mashima *et al*, 2009; Menendez & Lupu, 2007)). The co-localization of Mb with fatty acid synthase (FASN) we discovered might give a hint towards the fatty acid binding function of tumor Mb. Fatty acid binding properties of Mb have been reported and predicted early (Gloster & Harris, 1977; Lewis *et al*, 1968) and have recently gained further attention (Sriram *et al*, 2008). According to Flögel and colleagues, lack of Mb in the heart of knockout mice leads to a biochemical shift in cardiac substrate utilization from fatty acid to glucose oxidation which, not only corresponds to an adaptive reduction in O_2 consumption for the equimolar production of ATP, but more so implicates the protein in providing fatty acid substrates for the mitochondrial β-oxidation breakdown *in vivo* (Flögel *et al*, 2005). Our indirect demonstration, that Mb is likely to be regulated by intracellular fatty acid levels as shown by the inhibition of fatty acid synthase (FASN) now indicates a putative role for Mb in fatty acid metabolism of cancer cells and clearly warrants further study. Analysing the metabolomic profiles of Mb positive and negative breast cancer cases should also yield relevant data to shed more light on the putative role of Mb in fatty acid metabolism.

In summary, Mb is endogenously expressed in normal breast tissue and abundantly in a subset of breast cancer cases. The strong association of Mb expression with presence of the estrogen receptor (ERα) explains the generally better prognosis of Mb positive tumors, compared to tumors lacking Mb. It appears, that the regulation of Mb in tumors is so far only incompletely understood. Our data suggests that in breast cancer cells, Mb expression is regulated by estrogen signaling, possibly also by fatty acid levels and hypoxia. Mb`s prospective role in the lipid metabolism of ER-positive tumors provides a reasonable rationale to investigate this association in further studies, yet the role of

other stimuli, e.g. nitric oxide or growth factors also needs to be looked at in greater detail. Taken together, these findings further broaden our view on the role of non-muscle Mb that may have fundamental implications for our conception of the biology of solid tumors.

5. Acknowledgements

The excellent technical assistance of Britta Beyer, Annette Bohnert, Martina Bimmler, Martina Storz, Silvia Behnke, Sonja von Serenyi and Daniela Wichmann is thankfully acknowledged. We thank also thank Katharina Spanaus (Institute of Clinical Chemistry, USZ) for help and advice. Parts of this work were funded (J.H.) by the Swiss National Science Foundation and by a grant of the Faculty of Medicine (E.D.), RWTH Aachen (START network on tumor marker and their function).

6. References

Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA, Haroon ZA (2002) Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* **82:** 911-8

Bhargava R, Beriwal S, Dabbs DJ, Ozbek U, Soran A, Johnson RR, Brufsky AM, Lembersky BC, Ahrendt GM Immunohistochemical surrogate markers of breast cancer molecular classes predicts response to neoadjuvant chemotherapy: a single institutional experience with 359 cases. *Cancer* **116:** 1431-9

Brown JM (1999) The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. *Cancer Res* **59:** 5863-70

Brown JM, Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer* **4:** 437-47

Brunori M (2001) Nitric oxide moves myoglobin centre stage. *Trends Biochem Sci* **26:** 209-10

Cheng G, Li Y, Omoto Y, Wang Y, Berg T, Nord M, Vihko P, Warner M, Piao YS, Gustafsson JA (2005) Differential regulation of estrogen receptor (ER)alpha and ERbeta in primate mammary gland. *J Clin Endocrinol Metab* **90:** 435-44

Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T (2003) Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. *Proc Natl Acad Sci U S A* **100:** 13994-9

Elston EW, Ellis IO (1993) Method for grading breast cancer. *J Clin Pathol* **46:** 189-90

Eusebi V, Bondi A, Rosai J (1984) Immunohistochemical localization of myoglobin in nonmuscular cells. *Am J Surg Pathol* **8:** 51-5

Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, Macgrogan G, Bergh J, Cameron D, Goldstein D, Duss S, Nicoulaz AL, Brisken C, Fiche M, Delorenzi M, Iggo R (2005) Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene* **24:** 4660-71

Flögel U, Gödecke A, Klotz LO, Schrader J (2004) Role of myoglobin in the antioxidant defense of the heart. *FASEB J* **18:** 1156-8

Flögel U, Laussmann T, Gödecke A, Abanador N, Schafers M, Fingas CD, Metzger S, Levkau B, Jacoby C, Schrader J (2005) Lack of myoglobin causes a switch in cardiac substrate selection. *Circ Res* **96:** e68-75

Flögel U, Merx MW, Gödecke A, Decking UK, Schrader J (2001) Myoglobin: A scavenger of bioactive NO. *Proc Natl Acad Sci U S A* **98:** 735-40

Flonta SE, Arena S, Pisacane A, Michieli P, Bardelli A (2009) Expression and functional regulation of myoglobin in epithelial cancers. *Am J Pathol* **175:** 201-6

Galluzzo M, Pennacchietti S, Rosano S, Comoglio PM, Michieli P (2009) Prevention of hypoxia by myoglobin expression in human tumor cells promotes differentiation and inhibits metastasis. *J Clin Invest* **119:** 865-75

Garry DJ, Ordway GA, Lorenz JN, Radford NB, Chin ER, Grange RW, Bassel-Duby R, Williams RS (1998) Mice without myoglobin. *Nature* **395:** 905-8

Gloster J, Harris P (1977) Fatty acid binding to cytoplasmic proteins of myocardium and red and white skeletal muscle in the rat. A possible new role for myoglobin. *Biochem Biophys Res Commun* **74:** 506-13

Gödecke A, Flögel U, Zanger K, Ding Z, Hirchenhain J, Decking UK, Schrader J (1999) Disruption of myoglobin in mice induces multiple compensatory mechanisms. *Proc Natl Acad Sci U S A* **96:** 10495-500

Gray LH, Conger AD, Ebert M, Hornsey S, Scott OC (1953) The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol* **26:** 638-48

Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT, Kelm M, Rassaf T (2008) Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* **105:** 10256-61

Iseki M, Tsuda N, Kishikawa M, Shimada O, Hayashi T, Kawahara K, Tomita M (1990) Thymolipoma with striated myoid cells. Histological, immunohistochemical, and ultrastructural study. *Am J Surg Pathol* **14:** 395-8

Jamieson S, Rudland PS (1990) Identification of metaplastic variants generated by transfection of a nonmetastatic rat mammary epithelial cell line with DNA from a metastatic rat mammary cell line. *Am J Pathol* **137:** 629-41

Jürgens KD, Peters T, Gros G (1994) Diffusivity of myoglobin in intact skeletal muscle cells. *Proc Natl Acad Sci U S A* **91:** 3829-33

Kendrew JC (1958) Architecture of a protein molecule. *Nature* **182:** 764-7

Khan KK, Mondal MS, Padhy L, Mitra S (1998) The role of distal histidine in peroxidase activity of myoglobin--transient-kinetics study of the reaction of H2O2 with wild-type and distal-histidine-mutanted recombinant human myoglobin. *Eur J Biochem* **257:** 547-55

Kristiansen G, Fritzsche FR, Wassermann K, Jager C, Tolls A, Lein M, Stephan C, Jung K, Pilarsky C, Dietel M, Moch H (2008) GOLPH2 protein expression as a novel tissue biomarker for prostate cancer: implications for tissue-based diagnostics. *Br J Cancer* **99:** 939-48

Kuhajda FP, Pizer ES, Li JN, Mani NS, Frehywot GL, Townsend CA (2000) Synthesis and antitumor activity of an inhibitor of fatty acid synthase. *Proc Natl Acad Sci U S A* **97:** 3450-4

Kurebayashi J (2003) Endocrine-resistant breast cancer: underlying mechanisms and strategies for overcoming resistance. *Breast Cancer* **10:** 112-9

Lewis UJ, Cheever EV, Seavey BK (1968) Influence of fatty acids on the electrophoretic behavior of proteins with special reference to pituitary hormone and thyroglobulin. *J Biol Chem* **243:** 260-7

Mashima T, Seimiya H, Tsuruo T (2009) De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy. *Br J Cancer* **100:** 1369-72

Massaad-Massade L (2004) [Hypoxia, HIF1alpha and estrogen receptor]. *Bull Cancer* **91:** 677-83

Masuda K, Truscott K, Lin PC, Kreutzer U, Chung Y, Sriram R, Jue T (2008) Determination of myoglobin concentration in blood-perfused tissue. *Eur J Appl Physiol* **104:** 41-8

Menendez JA, Lupu R (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer* **7:** 763-77

Nitta T, Xundi X, Hatano E, Yamamoto N, Uehara T, Yoshida M, Harada N, Honda K, Tanaka A, Sosnowski D, Chance B, Yamaoka Y (2003) Myoglobin gene expression attenuates hepatic ischemia reperfusion injury. *J Surg Res* **110:** 322-31

Ordway GA, Garry DJ (2004) Myoglobin: an essential hemoprotein in striated muscle. *J Exp Biol* **207:** 3441-6

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* **406:** 747-52

Ruck P, Horny HP, Greschniok A, Wehrmann M, Kaiserling E (1995) Nonspecific immunostaining of blast cells of acute leukemia by antibodies against nonhemopoietic antigens. *Hematol Pathol* **9:** 49-56

Smith TW, Davidson RI (1984) Medullomyoblastoma. A histologic, immunohistochemical, and ultrastructural study. *Cancer* **54:** 323-32

Sriram R, Kreutzer U, Shih L, Jue T (2008) Interaction of fatty acid with myoglobin. *FEBS Lett* **582:** 3643-9

Theurillat JP, Ingold F, Frei C, Zippelius A, Varga Z, Seifert B, Chen YT, Jager D, Knuth A, Moch H (2007) NY-ESO-1 protein expression in primary breast carcinoma and metastases: correlation with CD8+ T-cell and CD79a+ plasmacytic/B-cell infiltration. *Int J Cancer* **120:** 2411-7

Thews O, Koenig R, Kelleher DK, Kutzner J, Vaupel P (1998) Enhanced radiosensitivity in experimental tumours following erythropoietin treatment of chemotherapy-induced anaemia. *Br J Cancer* **78:** 752-6

Thomlinson RH, Gray LH (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer* **9:** 539-49

Vaupel P, Schlenger K, Knoop C, Hockel M (1991) Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. *Cancer Res* **51:** 3316-22

Vaupel P, Schlenger KH, Hoeckel M, Okunieff P (1992) Oxygenation of mammary tumors: from isotransplanted rodent tumors to primary malignancies in patients. *Adv Exp Med Biol* **316:** 361-71

Veeck J, Niederacher D, An H, Klopocki E, Wiesmann F, Betz B, Galm O, Camara O, Durst M, Kristiansen G, Huszka C, Knuchel R, Dahl E (2006) Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis. *Oncogene* **25:** 3479-88

Wittenberg BA, Wittenberg JB, Caldwell PR (1975) Role of myoglobin in the oxygen supply to red skeletal muscle. *J Biol Chem* **250:** 9038-43

Wittenberg JB (1970) Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle. *Physiol Rev* **50:** 559-636

Yackzan KS, Wingo WJ (1982) Transport of fatty acids by myoglobin- a hypothesis. *Med Hypotheses* **8:** 613-8

Yang GC, Yee HT, Waisman J (2003) Metaplastic carcinoma of the breast with rhabdomyosarcomatous element: aspiration cytology with histological, immunohistochemical, and ultrastructural correlations. *Diagn Cytopathol* **28:** 153-8

Zhang PJ, Goldblum JR, Pawel BR, Fisher C, Pasha TL, Barr FG (2003) Immunophenotype of desmoplastic small round cell tumors as detected in cases with EWS-WT1 gene fusion product. *Mod Pathol* **16:** 229-35

Table 1 Clinico-pathological parameters of invasive breast cancer cases and relation to myoglobin expression

Characteristic	Number of cases (%)	MB negative	MB weak	MB moderate	MB strong	p-value
	917 (100%)					
<60 years	416 (45.4%)	125	132	130	29	0.170[#]
>=60 years	501 (54.6%)	142	157	142	60	
Pre-menopausal	198 (21.5%)	67	64	60	7	0.007[#]
Post-menopausal	719 (78.5%)	200	225	212	82	
Invasive ductal	739 (80.6%)	214	221	228	76	0.375[*]
Invasive lobular	125 (13.6%)	39	49	29	8	
NOS	53 (5.8%)	14	19	15	5	
pT1	335 (36.5%)	91	108	106	30	0.479[#]
pT2	410 (44.7%)	115	134	119	42	
pT3	66 (7.2%)	27	19	17	3	
pT4	106 (11.6%)	34	28	30	14	
pN0	346 (42.5%)	92	107	111	36	0.355[#]
pN1	369 (45.3%)	113	120	105	31	
pN2	69 (8.5%)	21	20	16	12	
pN3	31 (3.8%)	10	8	12	1	
G1	126 (13.7%)	30	40	46	10	0.001[#]
G2	460 (50.2%)	112	155	141	52	
G3	331 (36.1%)	125	94	85	27	
ER-negative	163 (18.7%)	78	45	34	6	0.001[#]
ER-Positive	709 (81.3%)	171	226	231	81	
PR-negative	314 (34.8%)	114	104	82	14	0.001[#]
PR-positive	588 (65.2%)	145	183	187	73	
HER2 0, 1+,2+,	776 (88.1%)	223	241	239	73	0.346[#]
HER2 3+	105 (11.9%)	33	37	25	10	
Ki-67 <=10%	466 (54%)	132	149	139	46	0.985[#]
Ki-67 >10%	393 (46%)	117	115	121	40	
CK5/6 neg.	793	212	260	239	82	0.001[#]
CK5/6 pos.	96	45	22	26	3	

[*] Pearson Chi-Square, [#] Chi-Square for trends

Table 2 Correlation of Myoglobin expression to endogenous Markers of hypoxia (CC; p-value)

	HIF-1α	**HIF-2α**	**GLUT1**	**CAIX**
Mb	0.095; 0.276	0.293; 0.001	-0.039; 0.64	0.286; 0.001
HIF-1α		0.215; 0.020	0.334; 0.001	0.285; 0.001
HIF-2α			-0.206; 0.024	0.223; 0.015
GLUT1				0.252; 0.003

Figure legends

Figure 1. Detection of Mb mRNA and protein in clinical breast cancer samples and cell lines

A) Myoglobin mRNA expression in ten randomly chosen samples of human breast cancer and corresponding normal breast tissues (#1 to #10) as measured by quantitative RT-PCR. Calculation of error bars according to Applied Biosystems user manual. Median myoglobin expression was up to 352-fold up-regulated in breast tumors (gray) compared to matching normal breast tissues (white). **B**) Myoglobin mRNA expression in benign MCF12A breast cells and various breast cancer cell lines. **C**) Normal lobular parenchyma of the breast, illustrating a mild Mb immunoreactivity in luminal epithelial cells (inner layer, thin arrow), whereas myoepithelial cells (outer cell layer, arrowhead) are negative. Note, that single luminal cells (bold arrow) are highlighted by a stronger Mb immunostaining (400x). **D**) Strong myoglobin positivity in a ductal carcinoma *in situ* (DCIS), low grade, showing a slight accentuation of staining in the center in support of O_2-diffusion gradients contributing to the Mb expression profile in tissues (200x). **E/F**) Examples of invasive ductal carcinoma of the breast (all 200x). **E**) Moderately differentiated with a moderate to strong and patchy Mb expression. Note also the central normal duct (arrow) with the Mb positive secretory cell layer and the Mb negative myoepithelial layer. **F**) Strong Mb immunoreactivity in a well differentiated carcinoma.

Figure 2. Mb expression is linked to better prognosis and ER-positivity and inversely related to estrogen concentration

A) Kaplan Meier analysis of a cohort of 917 primary breast cancer cases showing that tumors with high Mb expression (Mb+) demonstrate a significant prognostic value for the patient of an improved cumulative overall survival compared to cases with low Mb expression (Mb-). **B**) Myoglobin expression in MCF7 breast cancer cells is repressed by increasing concentrations of 17-ß-estradiol (dissolved in ethanol) as measured by quantitative RT-PCR. **C**) normal breast tissue (left) shows a weak immunofluorescence for Mb in the secretory cell layer, of which some stronger staining cells are intermingled. **D**) The immunofluorescence for ERα (left) shows a nuclear staining also of single cells. **E**)The merged Figure (left) illustrates, that these cells highlighted by ERα and Mb are

mostly corresponding (all 400x). **C-E**) This co-expression is also shown in a well differentiated invasive ductal breast carcinoma (panel on right hand side) (all 200x).

Figure 3. Double immunofluorescence (Mb/FASN) staining of breast tissues and regulation of Mb by inhibition of FASN

A) Normal breast tissue (panel on the left) shows weak immunofluorescence for Mb in the secretory cell layer, along with some stronger staining cells are intermingled. **B**) The immunofluorescence for FASN (middle) shows strong cytoplasmic staining. **C**) The overlay Figure (bottom) illustrates high degree of correspondence (yellow) between cells expressing Mb and FASN (all 400x). In invasive carcinomas (panel on the right), a greater variation is seen and co-expression of Mb and FASN is only preserved in a subset of cells as the overlay illustrates. In general, the FASN expression exceeds Mb immunoreactivity (all 200x). **D**) RT-PCR evidence for C75-mediated downregulation of Mb mRNA (left). Western blot evidence for C75-mediated downregulation of Mb protein (right).

Supplemental Legends.

Fig. S1. Establishment of a sensitive and specific immunohistochemistry protocol to detect myoglobin

Three different myoglobin antibodies were compared for their suitability to detect myoglobin in formalin fixed paraffin embedded (FFPE) tissues, i.e. mouse monoclonal clone z001, Zymed, USA; mouse monoclonal clone MG1, Neomarkers, USA and rabbit polyclonal anti-human myoglobin, DAKO, Denmark. All three antibodies yielded comparable results with clone z001 showing the cleanest immunoreactivity. To further enhance sensitivity a heat induced epitope retrieval (HIER) step was included in the z001 detection protocol (left). A blocking experiment with purified human myoglobin (1:10 mol) confirmed the specificity of the immunoreaction (right).

Fig. S2. Mb immunohistochemistry – distribution of expression during tumor progression

The distribution of intensity of Mb immunoreactivity, categorized as negative (green), weakly (yellow), moderately (orange) and strongly positive (red) in normal breast tissues, DCIS, invasive breast cancer and recurrences of invasive breast cancer is shown. Clearly, on average no simple down or upregulation ob Mb expression is noted, but a polarization with increasing losses and gains in intensity of Mb expression with tumor progression is apparent.

Fig. S3. Transmission electron microscopy of two Mb positive breast cancer specimens

Since myoglobin has been described as a marker of rhabdomyoid differentiation, two breast tumors with strong myoglobin immunoreactivity were analysed by transmission electron microscopy. **A)** Cell of an invasive lobular carcinoma with broad bundles of intermediate filaments (arrow). **B)** Cell of an invasive ductal carcinoma exhibiting a plethora of endoplasmatic reticulum. No striated muscle elements as exemplified in **C)** (human heart muscle) were noted.

Fig. S4. Kaplan Meier Analysis of overall survival of 872 breast cancer patients with respect to Mb and ERα status

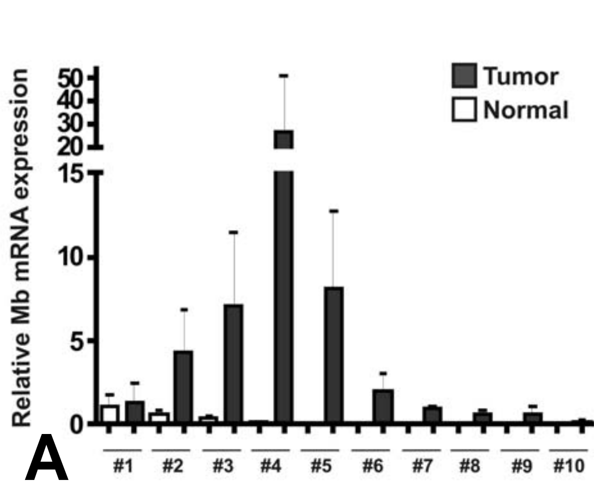
This figure illustrates the additive prognostic value of Mb and ER by comparing tumors negative for ER and MB (half-dotted line), tumors positive for ER and Mb (full line) and

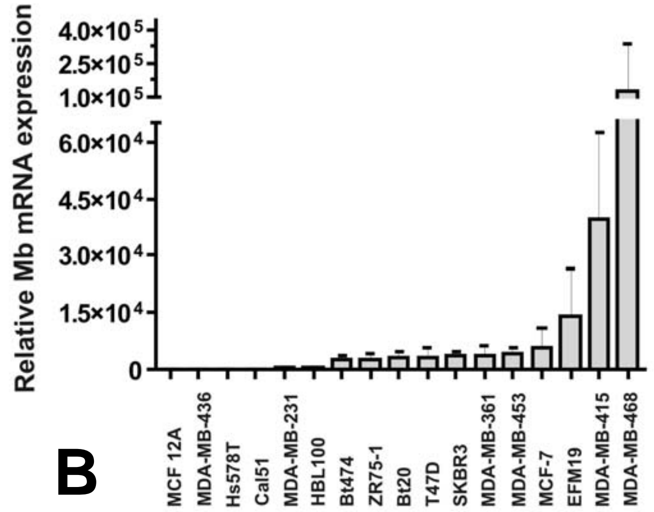
tumors which are positive for either, ER or Mb (dotted line). Note, that the latter group that has positivity for one marker indicative for luminal phenotype does do considerably better than tumors negative for both, however, it does not quite reach the relatively favourable course of the group in which both markers are positive.

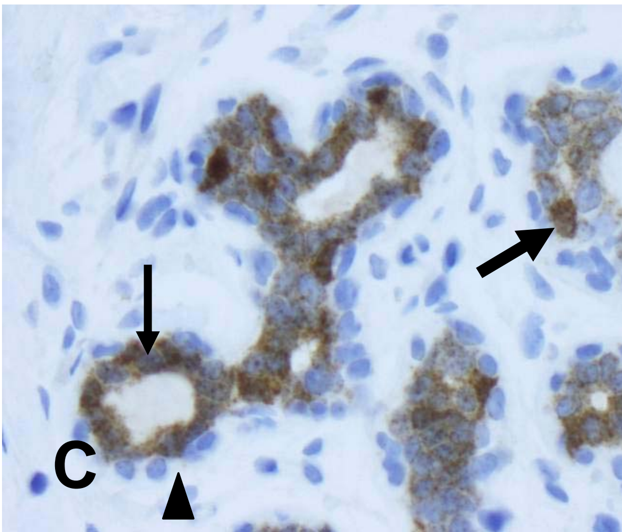
Fig. S5. In silico analysis of the influence of estrogen signaling on Mb and ESR1 in MCF-7 cells

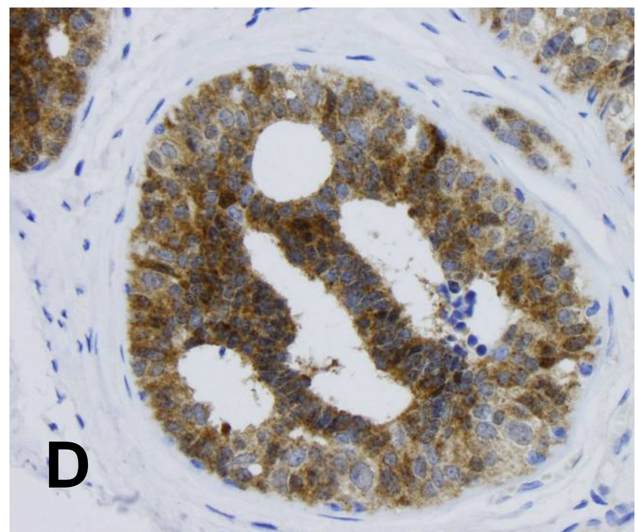
Retrieving the array datasets of Coser *et al.* (2003) via the web based data mining tool GEO Profiles, two experiments were analysed. On the left, the influence of estrogen starvation on the expression of Mb and ESR1 (=ERα) is illustrated. Clearly, a concomitant up-regulation of both transcripts (single channel counts, red bars) during 48h is apparent. Conversely, the profiles of MCF-7 cells that were treated with 17-β-estradiol (on the right) show a dose dependent down-regulation of Mb and ESR1. Note, however, that the amplitude of either ESR1 regulation (red bars) is artificially enhanced due to the start of Y-axis labels at non-zero values (explains poor correspondence with blue percentile rank data in ESR1 profiles).

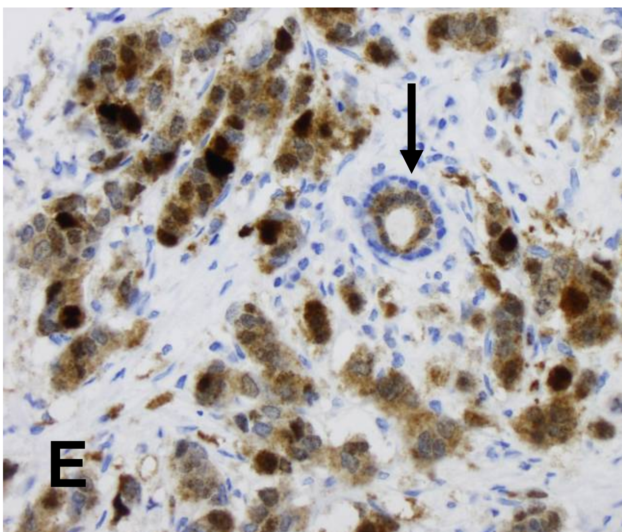
Figure 1











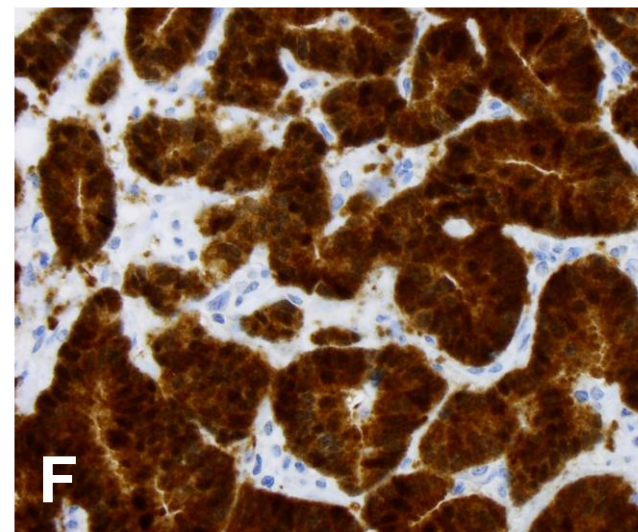
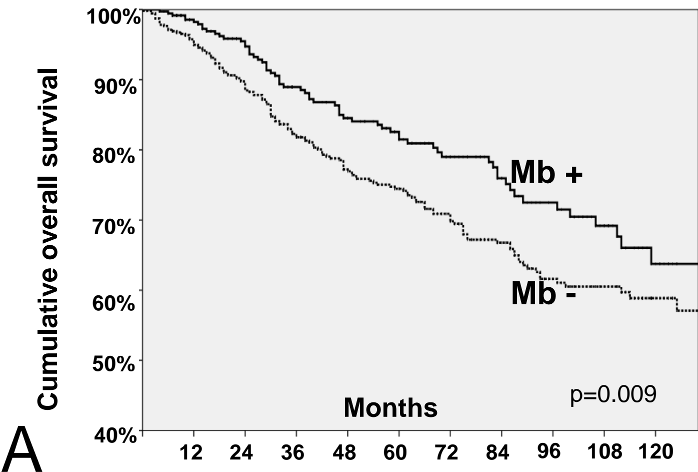
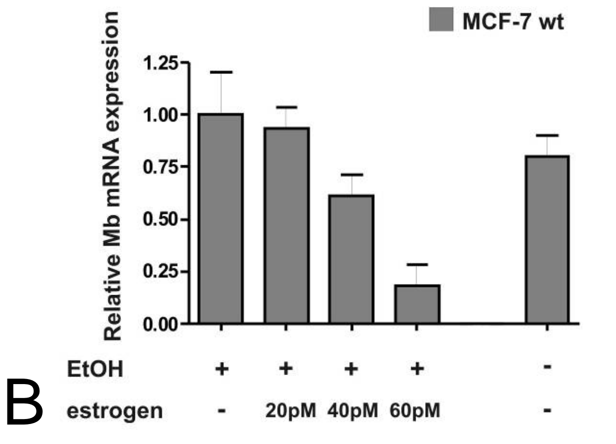
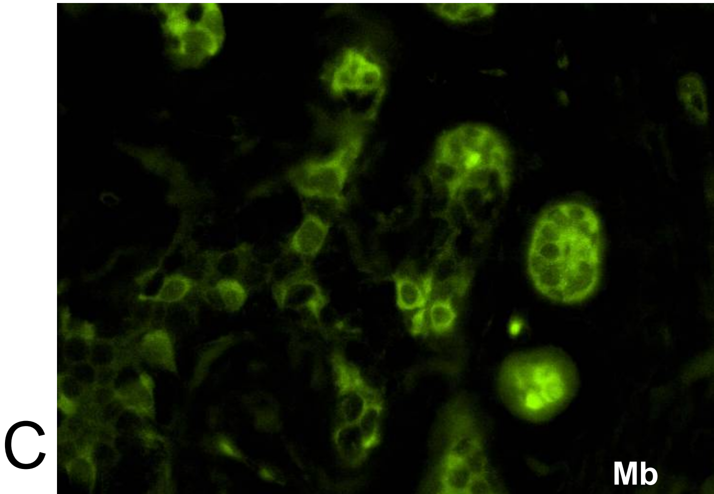
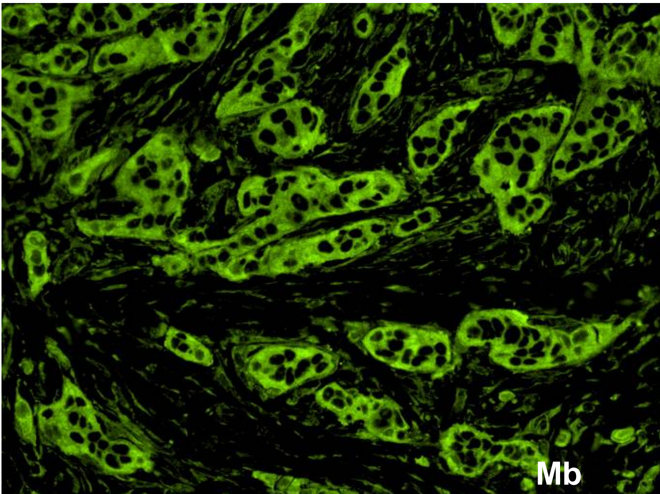


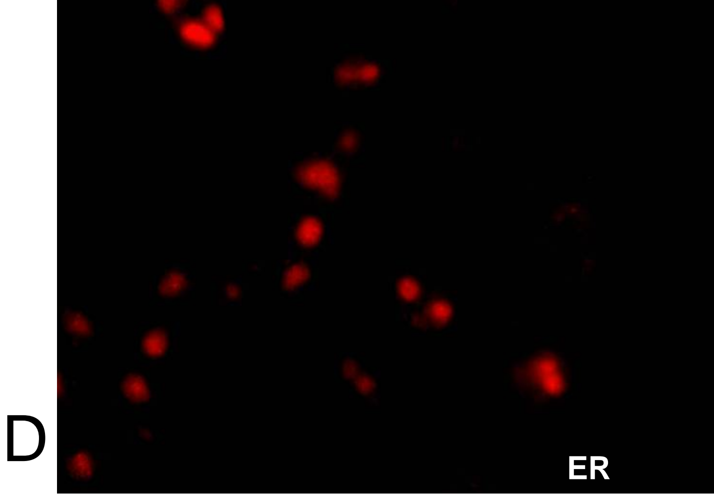
Figure 2

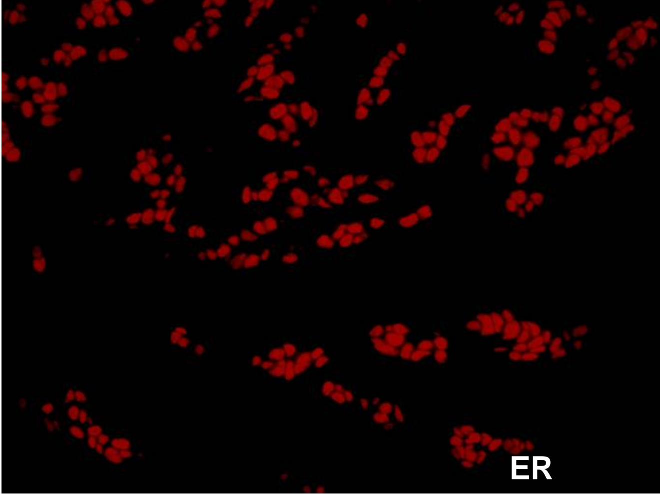


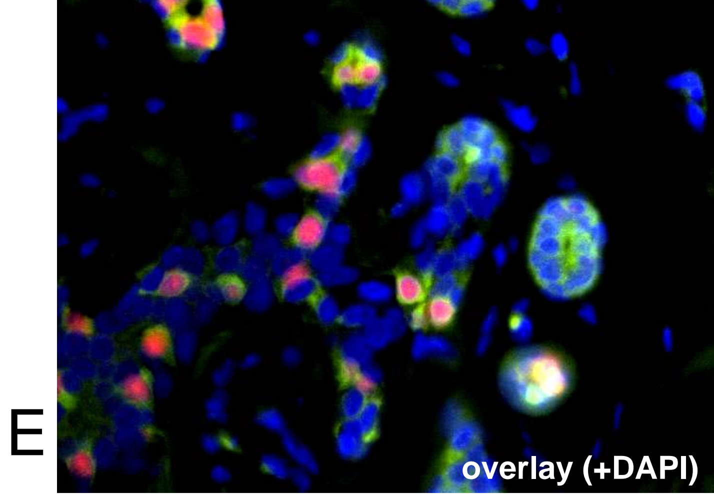












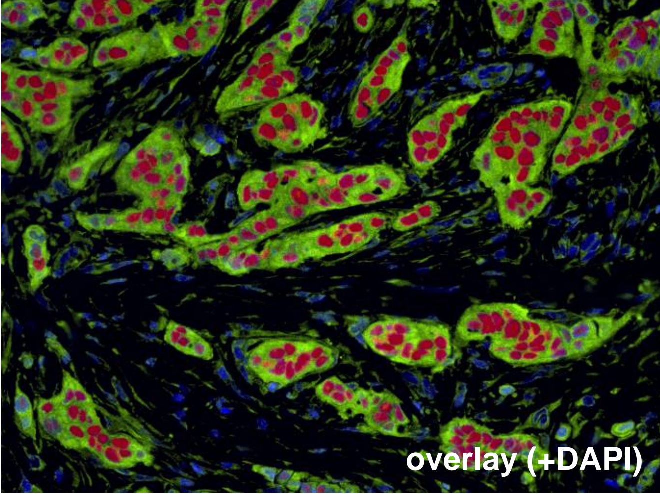


Figure 3

A

Mb

Mb

B

FASN

FASN

C

overlay (+DAPI)

overlay (+DAPI)

D