Tumor oxygenation

- *influence on mammary tumor growth, progression and response to chemotherapy*

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Dissertation for the degree Philosophiae Doctor (PhD)

at the University of Bergen

2011

Dissertation date: 28.04.2011

Contents

Acknowledgements

This thesis is based on work carried out at the Department of Biomedicine, University of Bergen (UoB), during the period from 2008-2011. The study has been financially supported by Helse Vest.

First and foremost I want to thank my supervisor, Linda Stuhr, for her unique participation and support. Your efficiency, availability and optimism have been invaluable for me. Daily mails or phone calls with encouragement have been greatly appreciated, especially the last six months. I also wish to express my gratitude to my co-supervisor Rolf Reed, for good advice and guidance throughout this project. Your encouraging pep-talks always cheer me up.

I also would like to thank past and present colleagues in the Heart and Circulation group. A special thanks go to my regular lunch-mates for hours and hours of discussions involving science, and everything but science. I would further acknowledge Gerd Salvesen for her help in the laboratory. It is a great pleasure to work with you and learn from your experience, both in the lab and in life in general. I also want to thank Odd Kolmannskog and Åse Eriksen for excellent technical assistance and for never saying no. To the fabulous post doc`s Åsa, Tine and Cecilie: Thanks for all your help and patience. Charlotte and Guro: I miss you every single day.

Further, I would like to thank my co-authors Charlotte Jevne, Odd Kolmannskog, Gerd Salvesen, Anne Øyan, Karl-Henning Kalland, Karl Johan Tronstad, Jian Wang, Lars Akslen, Martha Chekenya, Per Øystein Sakariassen and Linda Sleire for all their help. I especially want to acknowledge Anne Øyan for her enormous contribution to this project.

Thanks to all my friends, for always being there when I need it; I do not know what I would do without you. A special thanks to all the “BSI-oldies” for all the good times we had, and still have. My Bergen-years would never be the same without you. Also,

thanks to the members of AK Bjørgvin, for providing me with crazy stories, and for frequently testing my temper. To the Fab 4, Ann Margaret, Elisabeth, May-Lill and Ragnhild: “Blod og svette, slit og tåre, Julsundet vil alltid score!”. To Kjersti, for being my personal, fully automatic dishwasher, for gossiping, laughing and just being a good friend. To Harriet for making me honorary member of “flate rompers klubb”, and for being awesome. To Eli for four fantastic years as roommates and for being my pointer in what is right and wrong in life. To Gro, my big sis in Bergen, for sharing beautiful Olai with me. To Bjørn, for always yodelling and throat singing me into a better mood. To Anders, for being my favourite cousin, cop and person. My msn-friends also deserve greetings for allowing me to disturb them on a regular basis.

Last but not least, I want to thank my dear family, for supporting me and encouraging me, without interfering with my choices. To my father for enduring living so many years with four women, and for having the world’s biggest heart. To my mother for being perfect. And last but not least, a big thanks to my sisters for always making my life easier by clearing my path and watching my back. I am proud to be your little sister.

Bergen, 2011

Ingrid

Abbreviations

A: Surface area

ATP: Adenosine triphosphate

ARNT: Aryl hydrocarbon receptor nuclear translocator

Atm: Atmospheric pressure

BRCA1/3: Breast cancer type 1/3 susceptibility protein

C: Concentration of the solute gas

CAF: Cancer-associated fibroblast

CAM: Cell adhesion molecule

CFC: Capillary filtration coefficient

COP_c: The capillary colloid osmotic pressure

COP_{if}: The interstitial colloid osmotic pressure

D: Diffusion coefficient

DMBA: Dimethyl-α-benzanthracene

eGFP: Enhanced green fluorescent protein

ECM: Extracellular matrix

EMT: Epithelial-to-Mesenchymal Transition

ERα: Estrogen receptor α

FACS: Fluorescence-activated cell sorting

GLUT1/3: Glucose transporter 1/3

Hb: Hemoglobin

HBO: Hyperbaric oxygen

HIF: Hypoxia inducible factor

HRE: Hypoxia response element

J_s: Solute transport per unit time

J_v: Fluid filtration per unit time

K: A constant

kPa: Kilo Pascal

MET: Mesenchymal-to-Epithelial Transition

Msw: Meters sea water

NOD/SCID: Non-obese diabetic/severe combined immunodeficiency

P: Partial/Absolute gas pressure

P_c: Capillary hydrostatic pressure

PDGF: Platelet derived growth factor

P_{if} : Interstitial fluid pressure

pO_2: The partial pressure of oxygen, also called oxygen tension

pRB: Retinoblastoma protein

ROS: Reactive oxygen species

TGF-β: Transforming growth factor-β

UHMS: The Undersea and Hyperbaric Medical Society

V: Volume

VEGF: Vascular endothelial growth factor

VHL: Von Hippel-Lindau protein

α: The solubility coefficient

σ: The colloid osmotic reflection coefficient

ΔC: The concentration difference

Δx: Distance

2,3-DPG: 2,3-Diphosphoglycerate

5FU: 5-fluorouracil/

[H^3]-5FU: [H^3]-5-fluorouracil

List of Publications

This thesis is a summary of the following papers, which are referred to by their roman numerals in the text.

I. **Moen I, Oyan AM, Kalland KH, Tronstad KJ, Akslen LA, Chekenya M, Sakariassen PO, Reed RK, Stuhr LE (2009).** Hyperoxic treatment induces Mesenchymal-to-Epithelial Transition in a rat adenocarcinoma model. *PLoS One*, **4** (7):e6381.

II. **Moen I, Tronstad KJ, Kolmannskog O, Salvesen GS, Reed RK, Stuhr LE (2009).** Hyperoxia increases the uptake of 5-fluorouracil in mammary tumors independently of changes in interstitial fluid pressure and tumor stroma. *BMC Cancer*, **9**(1):446.

III. **Moen I, Jevne C, Wang J, Kalland KH, Chekenya M, Akslen LA, Sleire L, Enger PØ, Reed RK, Øyan AM, Stuhr LEB (2011).** Tumor-stroma interactions in 4T1 mammary tumors with and without enhanced oxygenation. *Manuscript.*

1. Introduction

1.1 Cancer Incidents

According to the Norwegian Cancer Registry, 26 121 new cases of cancer were recorded in Norway in 2008, of which 14 000 occurred in men and 12 121 in women. Cancers of the prostate, female breast, colon and lung are the most common cancers and comprise almost half of the total cancer incidents. Breast cancer remains the most frequent neoplasm in women, with 2 753 new cases in Norway in 2008 [1]. Fortunately, the introduction and improvement of breast cancer screening, together with better treatment, have reduced breast cancer mortality in western countries [2, 3]. Nevertheless, breast cancer still remains the leading cause of cancer mortality in women worldwide [4], with over 400 000 deaths per year, and further improvement of both detection and therapy is desirable and necessary.

Genetic factors, including the major susceptibility genes (BRCA1, BRCA2), may account for up to 10% of breast cancer cases in developed countries. However, the major factors influencing breast cancer risk are obesity, alcohol, exogenous hormones (oral contraceptives, hormone replacement therapy) and, possibly diet and lack of physical activity [4].

1.2 Tumor Biology

The word tumor is derived from the Latin word meaning “swelling”, and is a lesion formed by abnormal growth of cells. Tumor is, however, not synonymous with cancer and can be both benign and malign. Nevertheless, for the remaining part of this thesis, malignant tumors are referred to as tumors. Cancer is a group of diseases characterized by unregulated cell growth and the invasion and spread of cells from the site of origin to other sites in the body [5]. Cancers can grow in cell suspension (e.g. leukemia), but most grow as solid masses of tissue. The complexity of these

diseases has been, and still is, one of the biggest challenges for clinicians and cancer researchers. The complexity lies not only in the fact that there are different types of cancers, but within each type of cancer there is a great variation in both behaviour and responses to treatment.

1.2.1 Hallmarks of Cancer

Hanahan and Weinberg [6] suggested six molecular, biochemical and cellular traits that characterize the development and progression of malignant tumors, and called them jointly as "The six hallmarks of cancer". Through different mechanisms, this set of functional capabilities is acquired during the development of the cancers.

The six hallmarks are [6]:

1. *Growth signal autonomy*
   Oncogenes mimicking normal growth signalling and growth signals from the stromal cells make the cancer cells independent on external growth factor signalling to proliferate.
2. *Insensitivity to growth inhibitory signals*
   The main example is disruption of the retinoblastoma protein (pRB)-pathway in cancer cells liberates the E2F transcription factors and allows cell proliferation, even with anti-proliferative signalling.
3. *Evasion of apoptosis*
   Many changes can lead to acquisition of apoptotic resistance, but mutation in the tumor suppressor gene p53 is the most common one. Functional inactivation of the p53 protein destroys the DNA damage sensor that normally induces the apoptotic cascade, opening for maintenance of mutations.
4. *Unlimited replicative potential*
   Telomerase maintains the length of the chromosomes, the telomeres, making cancer cells avoid entering senescence and giving them an unlimited replicative potential.

5. *Angiogenesis*

   To be able to survive and grow, the cancer cells are dependent on formation of new blood vessels, angiogenesis. Alteration of the balance between angiogenic inducers and inhibitors can activate the 'angiogenic switch'.

6. *Invasion and metastasis*

   Normal cells maintain their location in the body, and generally do not migrate. Successful migration and invasion depend on alteration of the binding of cells to their surroundings, like changes in cell adhesion molecules (CAMs), and are also dependent on the capabilities of all the previously mentioned hallmarks. Migration and invasion of cancer cells to other parts of the body is the major cause of cancer deaths.

From the above, it is evident that it is important not to study tumor cells in isolation, but rather in a context of surrounding and infiltrating tissue; collectively known as the tumor stroma or the tumor microenvironment.

1.2.2 The Tumor Microenvironment

The solid tumor forms a highly complex tissue, comprising heterogeneous malignant cells, as well as normal- and cancer-associated fibroblasts (CAFs), immune cells, pericytes, endothelial cells and extracellular matrix (ECM) (Figure 1) [7].

In general, the tumor stroma is characterized by low proteoglycan and hyaluronan concentrations and absence of an anatomically well-defined lymphatic network [8]. Additionally, the structural network of a tumor contains a dense network of collagen fibres, which are thicker and more numerous than that of normal connective tissue, and results in a more rigid tumor tissue [8]. Cancer-associated fibroblasts, inflammatory cells and mediators infiltrate the tumor stroma. Together they secrete various cytokines, growth factors and hormones than can directly stimulate tumor cell proliferation, survival, angiogenesis, invasion and metastasis [7, 9, 10].

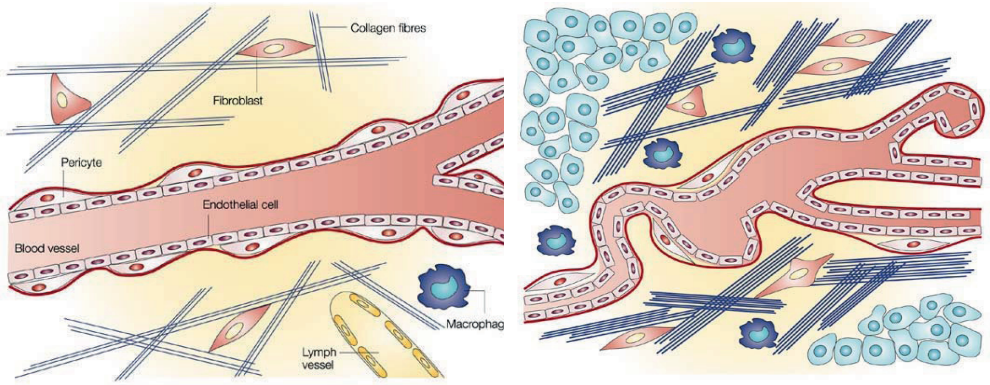

Figure 1: Anatomy of normal tissue (left panel) and tumor tissue (right panel). Tumor blood vessels are disorganized, tortuous and dilated, with uneven diameter and excessive branching and shunts, leading to inefficient blood flow. Furthermore, tumors contain a great number of infiltrating cancer-associated fibroblasts and inflammatory cells, in addition to the neoplasic tumor cells. The collagen network of solid tumors is more rigid than that of normal tissue and there is absence of an anatomically well defined lymphatic network. Adapted from Heldin *et al.* [11], with permission from Nature Publishing Group.

Tumors can not grow beyond a critical size or metastasize to other organs without blood vessels. Angiogenesis provides a principle mechanism for the maintenance of adequate blood flow in an expanding tumor tissue. Due to an imbalance in the angiogenic regulators, tumors have highly disorganized, tortuous and dilated blood vessels, with uneven diameter and excessive branching and shunts [12], leading to a highly variable blood flow. Together, these traits lead to inefficient and incomplete supply of nutrients and removal of waste products, and a hypoxic tumor microenvironment. Blood flow within a tumor is anything but uniform. Tumors contain both highly perfused areas, which are rapidly growing, and areas with reduced blood flow, which often are associated with development of necrosis [13]. In essence, the abnormal vasculature of tumors, result in an abnormal microenvironment, and together they form an obstacle to the delivery and efficacy of cancer therapy.

During cancer progression, genetically transformed cells (cancer cells) change the stromal host compartment, to form a permissive and supportive microenvironment for

further growth and progression [14]. Thus, it is now appreciated that the tumor microenvironment is crucial for cancer initiation, growth and progression, and that the interaction between the stromal components and the tumor cells is bidirectional [7, 15, 16].

1.2.3 Transcapillary Exchange

Transport of fluid and solute molecules in the interstitium, i.e. the extracellular fluid compartment between blood vessels and cells, is governed by the biological and physiochemical properties of the molecule and the properties of the ECM [8], and is an important feature influencing the tumors response to therapy. Transport of materials across the vessel wall is mainly governed by diffusion, i.e. solute transfer driven by a concentration gradient, as summarized in Fick`s law:

$$J_S = -DA\left(\Delta C / \Delta x\right)$$

Formula 1: Fick`s law

J_s= The mass of solute transferred by diffusion per unit time,
ΔC= The concentration difference across the capillary wall,
Δx= Distance (Thickness of the capillary wall), A= Surface area,
D= The diffusion coefficient (inversely related to solute size).

However, convection, i.e. solute flux carried by the fluid flux, also contributes to transvascular transport of materials. Transcapillary fluid balance aims to maintain constant fluid volume in the interstitium. The balance is determined by: 1) The properties of the capillary membrane, 2) The transcapillary hydrostatic pressure, 3) The transcapillary colloid osmotic pressure.

E. H. Starling described the relationship between these factors in 1896, and thus the factors influencing the transcapillary fluid flux are often referred to as the Starling equation [17]:

$$J_V = CFC\left[\left(P_c - P_{if}\right) - \sigma\left(COP_c - COP_{if}\right)\right]$$

Formula 2: The Starling equation.

Jv= Filtration, CFC= Capillary filtration coefficient, P_c= Capillary hydrostatic pressure, P_{if}= Interstitial fluid pressure, σ= The colloid osmotic reflection coefficient, COP_c= Colloid osmotic pressure (capillary), COP_{if}= Colloid osmotic pressure (interstitium)

The CFC is determined by the surface area of the capillary wall and its hydraulic conductivity. σ is the colloid osmotic reflection coefficient for proteins. The primary driving force for filtration is the capillary hydrostatic pressure (P_c). Filtration is on the other hand opposed by the colloid osmotic pressure of the plasma proteins (COP_c), which tends to hold the fluid in the circulation. COP_{if} is the colloid osmotic pressure tending to pull fluid out of the circulation. The interstitial fluid pressure (P_{if}) is the pressure exerted by the interstitium, which in skin normally varies between 0 and -2 mmHg [18]. This pressure is crucial in the control of a stable volume in the interstitium and is mainly determined by the capillary fluid filtration and the lymph flow, in other words the extracellular fluid volume. In addition, the forces governed by the structural network of the interstitium are a contributing factor in regulation of P_{if}, as determined by the interstitial pressure-volume relationship; the compliance [19]. It is now well established that P_{if} in most solid tumors is increased (up to ~60mmHg) [11]. An increase in P_{if} counteracts filtration, and has therefore been proposed as an obstacle to cancer therapy [11]. In Paper II we have studied the influence of P_{if} on the uptake of chemotherapy in the DMBA (dimetyl-α-benzanthracene)-induced mammary tumor model.

The mentioned features of the tumor biology influence growth and development of tumors, and its susceptibility to therapy. Furthermore, tumor hypoxia is an important factor, shared by most solid tumors, which will be thoroughly elucidated further in this thesis.

1.3 Tumor hypoxia

Hypoxia is defined as a reduction in the normal level of tissue oxygen. Tumor growth often overrides the ability of the tumor vasculature to adapt to the increasing oxygen demand. In addition, as already stated, tumor vasculature constitutes a fundamental difference between normal and tumor tissue, with its structural and functional abnormalities. Together, this often leads to solid tumors with areas subjected to acute or chronic hypoxia [20]. Although severe or prolonged hypoxia is toxic to both cancer cells and normal cells, adaptation to the hypoxic microenvironment has allowed cancer cells to survive and proliferate in this hostile milieu [21]. Traditionally, studies of tumor hypoxia were performed because of its proven effect on resistance to radiation therapy [22]. Later, Hockel *et al.* [23] showed that low oxygen tension in tumors was also associated with increased metastasis and poor survival rate. Michieli *et al.* [20] stated that the insufficient oxygen supply can limit tumor cell division, but on the other hand it can also select for more malignant cells and thereby lead to tumor progression. This "Darwinian selection" will lead to more aggressive cancer cells [20].

Hypoxia is known to result in cellular responses which improves oxygenation and viability through induction of angiogenesis, a raise in energy production by increased glycolytic metabolism and up-regulation of genes involved in cell survival/apoptosis (Figure 2) [24]. Hypoxia has also been shown to increase the genetic instability, activate invasive growth and persevere the undifferentiated cell state, which all in turn can be advantageous during tumor progression [20, 21].

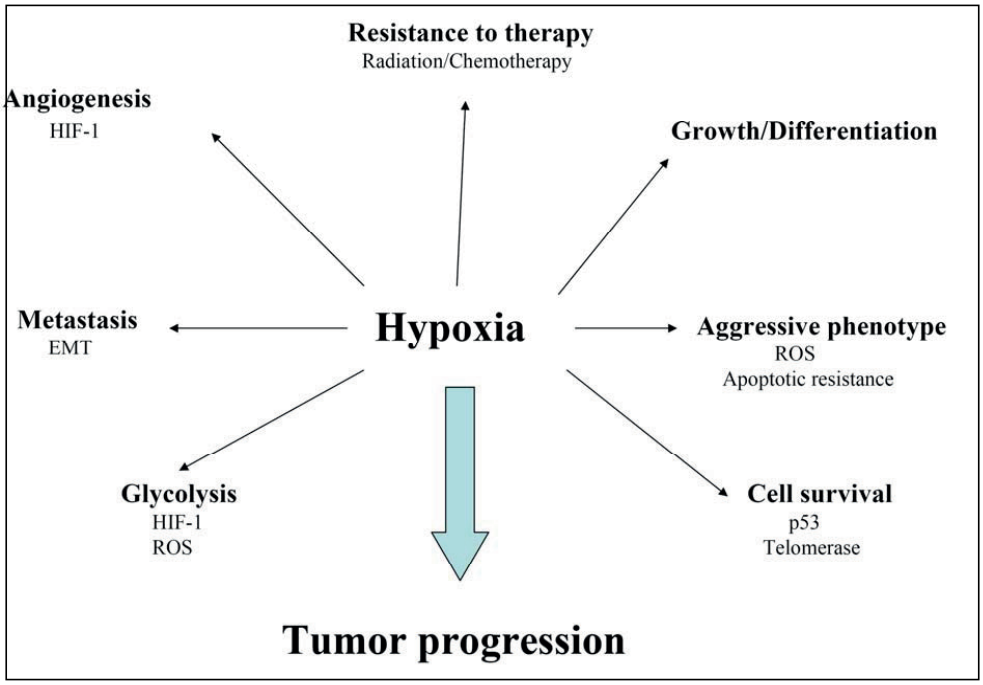

Figure 2: Tumors survive and progress under hypoxic conditions due to adaptations of cellular responses which improve oxygenation and viability through induction of angiogenesis, increase in glycolytic metabolism and up-regulation of genes involved in cell survival/apoptosis, leading to more aggressive and metastatic tumors. Hypoxia is also known to induce resistance to therapy. EMT: Epithelial-to-Mesenchymal transition, HIF-1: Hypoxia inducible factor 1, ROS: Reactive oxygen species.

1.3.1 Hypoxia and the Angiogenic Switch

The `angiogenic switch` involves up-regulation of pro-angiogenic factors and down-regulation of anti-angiogenic factors, causing formation of new blood vessels, although with variable degree of functionality. Generally, tumors cannot grow beyond ~ 1 mm in diameter without being supplied by new blood vessels [25]. Hypoxia is the most potent stimulator of angiogenesis. It is somehow paradoxical that the tumor tissue is hypoxic due to abnormal and non-functional tumor vasculature, as the hypoxic tumor tissue is responsible for inducing angiogenesis in the first place [26].

The hypoxia-inducible factor 1 (HIF-1)-pathway is one way that cells respond to reduced oxygen levels. HIF-1 is a transcription factor composed of HIF-1α and HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT) [27, 28]. The β-subunit is constitutively expressed, whereas the stability and activity of the α-subunit is inducible. The availability of the HIF-1α-subunit is primarily regulated by cellular oxygen levels [29], but also by growth factors [30]. Thus, in presence of oxygen, HIF-1α is bound to the tumor suppressor Von Hippel-Lindau (VHL) protein, causing ubiquitylation and degradation by the proteasome. In absence of oxygen, however, HIF-1α translocates to the nucleus, where it dimerizes with ARNT, binds to hypoxia-response elements (HRE's), and thereby activates the transcription of several hypoxia-response genes. Pro-angiogenic growth factors, like transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) are all induced by HIF-1, stimulating angiogenesis.

1.3.2 Hypoxia and Cell Survival

Normally, when mutations occur and DNA repair enzymes cannot correct the error, the apoptotic cascade is activated, leading to programmed cell death. However, the escape from this apoptotic program is one of the hallmarks of cancer, and tumor cells thereby maintain uncorrected mutations. The p53 tumor suppressor gene has been described as "the guardian of the genome", and can activate DNA repair enzymes, induce growth arrest and initiate apoptosis, and thereby protect cells against neoplastic transformation. Hypoxia is, in addition to DNA damage, able to stimulate p53 levels and activate the p53 protein [31]. However, deregulation and loss of function of p53 is seen in more than 50% of human cancers [31], and has proved to promote growth and malignant progression. Telomere length is an important factor deciding cell replicating ability, activation of p53 lead the cells into senescence when the telomeres have eroded. However, hypoxia has been shown to reduced telomere erosion by activation of telomerase, resulting in unlimited replicative potential [32]. Furthermore, Graeber *et al.* [33] showed that p53 mutation results in over-expression of the apoptotic inhibitor Bcl-2 and thus results in a substantial reduction of hypoxia-

induced cell death. Hypoxia thereby acts as a physiological selective agent, promoting the clonal expansion of apoptosis-resistant cells [33].

Additionally, Ravi *et al*. [34] showed that loss of p53 function in tumor cells enhances HIF-1α levels and increases the transcription of pro-angiogenic genes like VEGF in response to hypoxia.

1.3.3 Hypoxia and the Glycolytic Switch

Insufficient oxygen availability in tumors causes a shift in energy production from oxidative phosphorylation to anaerobic glycolysis to satisfy the tumor`s energy demand. Further, adaption to intermittent hypoxia leads to persistent metabolism of glucose to lactate even during aerobic conditions, a process called “the Warburg effect” [35]. Glycolysis leads to a lower ATP yield than oxidative phosphorylation, and thereby forces the tumors to increase their metabolic rate [13, 36]. Important components of the glycolytic pathways are the glucose transporters GLUT-1 and GLUT-3 and the enzyme hexokinase, all genes regulated by HIF-1, and thus by hypoxia [36]. It has also been showed that the end-products of glycolysis, lactate and pyruvate, regulate HIF-1 gene expression independently of hypoxia by stimulating the accumulation of HIF-1α, and thereby facilitating further glycolysis and tumor progression in a positive feedback loop [37].

Also, reactive oxygen species (ROS), or free radicals, are a by-product of cellular metabolism [38]. Oxidative stress, a result from imbalance between production of ROS and activation of the cell’s own antioxidant defence, is a phenomenon linked to carcinogenesis and several other chronic diseases [39]. In excess, ROS can cause lipid peroxidation, damage cell membranes, cause DNA damage and lead to cell death [38].

1.3.4 Hypoxia and Metastasis

Increased metastatic ability has been associated with hypoxic tumors [40, 41]. Evidence show that the effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced changes activating angiogenesis, glycolysis and inhibition of apoptosis, in addition to up-regulating growth factors and proteins involved in tumor invasiveness [42]. All these features enable tumor cells to survive or escape their hostile environment.

A key step in tumor metastasis is postulated to involve the de-regulation of tumor cell-cell and cell-ECM interactions through Epithelial-to-Mesenchymal transition (EMT) [26]. EMT is a fundamental process that governs morphogenesis in multicellular organisms [43]. However, in tumor progression, EMT is believed to have a more sinister role, leading to carcinomas with an invasive or metastatic phenotype (Figure 3) [43-45]. Hypoxia has been shown to trigger EMT, enabling cell detachment and invasion [46]. The “cadherin switch”, with down-regulation of E-cadherin (CDH1) and up-regulation of N-cadherin (CDH2) is one of the key regulators of EMT, as increased levels of CDH2 increase the motility and migration of the cells. Due to the morphological similarities of the primary tumors and the metastatic lesions, it has been hypothesized that tumors reactivate certain epithelial properties at the secondary site, through a Mesenchymal-to-Epithelial transition (MET) [45, 47]. However, this has yet to be proven. Tsuji *et al.* [48] have proposed an alternative hypothesis, where epithelial and mesenchymal cells cooperate to induce metastasis, and thus eliminates the need for MET at the distant site.

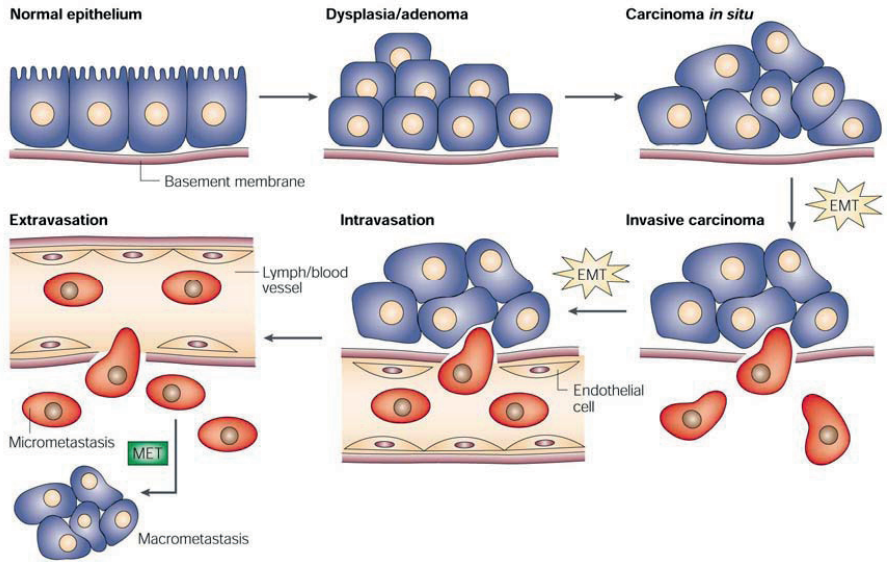

Figure 3: Proposed role of Epithelial-Mesenchymal-transition (EMT) and Mesenchymal-to-Epithelial-transition (MET) in cancer progression. Local proliferation and transformation of epithelial cells can lead to a carcinoma *in situ*. Further progression – possibly through EMT and fragmentation of the basement membrane - can lead to invasion into the lymph or blood vessels, and spread to distant organs. At secondary sites, carcinoma cells can extravasate and form solitary micrometastasis or form new carcinomas through MET. Adapted from Thiery *et al.* [43], with permission from Nature Publishing Group.

1.3.5 Hypoxia and Resistance to Therapy

Hypoxia can be a direct cause of resistance to conventional therapy. Grey *et al.* [22] proved in the 1950ies that oxygen concentration influence the effect of radiation therapy. Later, a number of factors associated with tumor hypoxia have also been directly or indirectly shown to influence the effect of chemotherapy [49]. The hypoxic therapy resistance has been ascribed to: 1) Some drugs and radiation requiring oxygen to generate ROS to be maximally cytotoxic. 2) Altered cellular metabolism reducing drug cytotoxicity. 3) Hypoxia leading to genetic instability, which can lead to more rapid development of drug-resistant cells [50]. Additionally, as for oxygen, the distribution of the chemotherapeutic drug is compromised due to the uneven blood flow in tumors and high distance from the nearest capillary [51].

1.4 Hyperbaric Oxygen Therapy

With our present knowledge of hypoxia and its effect on tumor growth, progression and response to chemotherapy, we aimed to investigate if a reduction in the hypoxic state of the mammary tumors, by enhancing tumor oxygenation, might have an inhibitory effect on tumor growth *per se*, in addition to enhancing the effect of chemotherapy.

Hyperbaric oxygen (HBO) is one way to increase tissue oxygenation. HBO therapy involves breathing of 100% oxygen pressurized to 2-3 times atmospheric pressure, equivalent to 10-20 meters sea water (msw). By taking advantage of the physical properties of gases under pressure, HBO expose tissues to elevated oxygen concentrations.

Henry`s law (Formula 3) states that the amount of a gas that will dissolve in a liquid at a given temperature is directly proportional to the partial pressure of the gas above the liquid, and of the solubility coefficient of the gas in the liquid. The solubility coefficient decreases with rising temperature and increasing salinity.

$$C = \alpha \times P$$

Formula 3: Henry`s law

C=The concentration of the solute gas, α= The solubility coefficient,
P= The partial pressure of the gas in the gas phase above the solution.

Boyle`s law (Formula 4) states that if the temperature of a fixed mass of gas is kept constant, the relationship between volume and pressure will vary in such way that the product of the pressure and volume will remain constant.

$$P \times V = K$$

Formula 4: Boyle`s law

P= The absolute pressure, V= The volume, K= A constant

Together, Boyle`s and Henry`s laws constitute the basis for hyperbaric oxygen therapy.

Increasing the partial pressure of oxygen (pO_2) has essentially no effect on the amount of oxygen bound to hemoglobin (Hb), as Hb is 96-98 % saturated at sea level in normal air, giving a O_2 content of approximately 200ml O_2/1000mL arterial blood. Additionally, 3.2 mL O_2/1000mL blood is dissolved in plasma and is carried in solution. The saturation/desaturation of dissolved O_2 strictly follows Henry`s law (Formula 3), while the O_2 saturation/desaturation of Hb is modified by the actual pH, CO_2, 2,3-diphosphoglycerate (2,3-DPG) and temperature, following a sigmoid curve. Therefore, treatment with HBO will hyper-saturate the blood with oxygen, as shown in Table 1.

Table 1: Theoretical arterial oxygen tensions pO_2 (mmHg) and dissolved O_2 concentrations for different normobaric and hyperbaric oxygen treatment protocols. All values assume arterial pO_2=alveolar O_2. Modified from Tarun *et al.* [52].

Depth	Pressure	%O_2	pO_2 (mmHg)	mL dissolved O_2/1000mL blood
0 m	1 bar	Normal air (~21%)	159	3.2
0 m	1 bar	100 %	760	20.9
10 m	2 bar	Normal air	318	8.1
10m	2 bar	100%	1520	44.4
15m	2.5 bar	Normal air	397.5	10.6
15m	2.5 bar	100%	1900	56.2
20m	3 bar	Normal air	477	13.1
20 m	3 bar	100 %	2280	68

Thus, at 3 bar pure oxygen, the amount of oxygen dissolved in the blood and delivered to the tissues is 20 times higher than during normal atmosphere, and is thereby sufficient to support resting tissue without the contribution of hemoglobin [53]. The dissolved oxygen can more easily reach areas where the red blood cells cannot pass, and can enable tissue oxygenation even with impaired hemoglobin oxygen carriage [53]. It has been shown that the distance that oxygen can diffuse through normal tissue is increased fourfold after a HBO treatment [54]. Thus, HBO

leads to increased oxygenation of tissue by increasing the transport of soluble oxygen. In general, if pressure does not exceed 3 bar and the length of therapy is less than 2 hours, HBO is considered safe [55].

The Undersea and Hyperbaric Medical Society (UHMS) has a list of approved indications for HBO-therapy (Table 2). HBO is the treatment of choice for decompression sickness, problem wounds and severe carbon monoxide poisoning. The duration of a single treatment (2.5-3 bar) varies from 45-90 min for carbon monoxide poisoning up to 2-4 h for some severe decompression disorders. For problem wounds most protocols average 90-120 min each of 20-30 treatments at 2.4 bar [56].

Table 2: Approved uses of hyperbaric oxygen (HBO) therapy as defined by the Undersea and Hyperbaric Medical Society (UHMS).

Air or gas embolism
Carbon monoxide poisoning
Central retinal artery occlusion
Clostridal myositis and myonecrosis
Crush injury, compartment syndrome and other acute traumatic ischemias
Decompression sickness
Enhancement of healing in selected problem wounds
Exceptional blood loss
Intracranial abscess
Necrotizing soft tissue infections
Osteomyelitis
Delayed radiation injury
Skin grafts and flaps
Thermal burns

1.5 History of HBO and Cancer

As in normal tissue, the pO_2 in tumor tissue increases significally during HBO exposure [57]. It has also been shown that the elevation of the tumor oxygen concentration is preserved for more than 60 min after HBO treatment [58].

The use of HBO in cancer management was controversial, and many had concerns that HBO would recruit hypoxic tumor cells into the pool of proliferative cells, initiate angiogenesis, and thereby lead to cancer progression. However, two extensive reviews have concluded that HBO do not promote tumor growth [59, 60]. To complement the reviews written by Daruwalla *et al.* [60] and Feldmeier *et al.* [59], I have reviewed the work performed on HBO and cancer the last 7 years (Table 4, Appendix). This mini-review support the previous findings [59, 60], as none of the reports observed a tumor-promoting effect of HBO (Table 4, Appendix). Nevertheless, as other treatment modalities, HBO display great variance in response between different tumor types.

Our group have tested the use of HBO treatment in both mammary tumors and gliomas *per se*, and found that HBO has a significant growth-inhibitory effect in both tumor models [61-63]. Thus, these studies formed the background for this thesis. Stuhr *et al*. [61] found that HBO both alone and in combination with the chemotherapeutic agent 5-fluorouracil (5FU) induced a clear reduction in tumor size compared to day 1 measurements in a chemically-induced (DMBA) mammary tumor model (Figure 4). The same study also included a group of rats exposed for a longer experimental period and with additional treatments (23 days, 7 treatments) [61]. The results showed that although the experimental period was prolonged with 12 days and three additional HBO treatments, no further reduction in tumor size was registered (Figure 5, series 6). Thus, the HBO effect was maximal after four HBO exposures. Furthermore, one series followed tumor growth 12 days post HBO treatment (Figure 5, series 5). This group showed that although HBO treatment was ended, the tumor size was significantly below day 1 level. This seems to imply that some permanent

changes must have occurred in the tumor tissue and that these changes prevent the tumor from further growth.

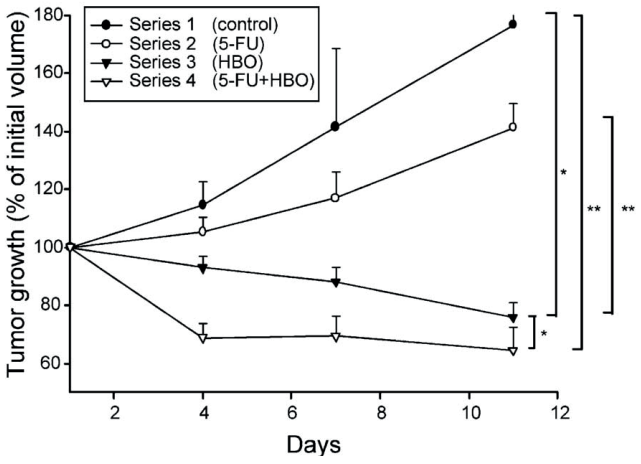

Figure 4: The effect of 5-fluorouracil (5FU) and hyperbaric oxygen (HBO) on DMBA-induced tumor growth - alone or in combination. Treatments are given day 1, 4, 7 and 10. Values represent means ± SE. *p<0.05 and **p<0.01. Adapted from Stuhr et al. [61].

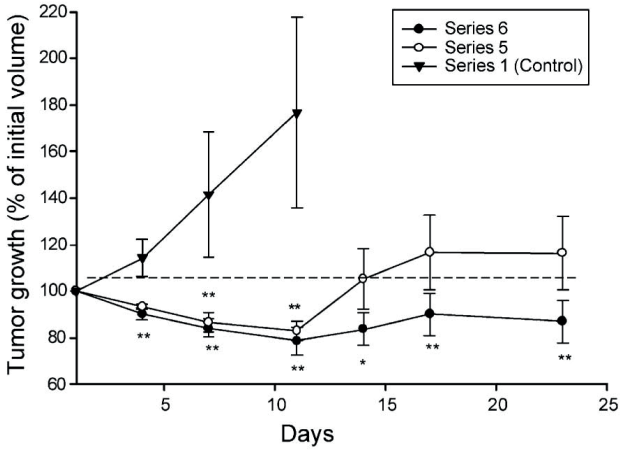

Figure 5: The effect of hyperbaric oxygen (HBO) on DMBA-induced tumor growth. The after-effect of 4 HBO exposures (Series 5) and the effect of multiple HBO exposures (Series 6). Dashed line indicates the level for initial tumor size. * p<0.05 vs day 1 and **p<0.01 vs day 1. Adapted from Stuhr *et al.* [61].

These results support the fact that HBO has a tumor-inhibitory effect on DMBA-induced mammary tumors *per se*. The possible use of HBO has a stand-alone- or adjuvant treatment in breast cancers is discussed in section 5.1 and 5.2.

2. Aims of Study

The overall aim of this thesis was to study the effect of enhanced oxygenation on mammary tumor growth, progression and response to therapy.

Specific aims

1) To investigate the effect of HBO therapy on tumor growth and progression in two different *in vivo* mammary tumor models, by elucidating cell proliferation, cell death and angiogenesis.

2) To study the gene expression profile of both untreated and HBO treated mammary tumors.

3) To develop a mammary tumor model, to be able to study tumor-stroma interactions, in addition to studying both aim 1 and 2.

4) To elucidate the effect of HBO on the uptake of a conventional chemotherapeutic drug, 5FU, into the mammary tumor tissue. Furthermore, to study possible oxygen-related changes occurring in the tumor stroma that might have influenced the chemotherapeutic response, as interstitial fluid pressure, amount of collagen, fluid distribution and reactive oxygen species.

3. Summary of Results

3.1 Paper I

Hyperoxic treatment induces Mesenchymal-to-Epithelial Transition in a rat adenocarcinoma model.

Tumor growth in the DMBA-induced tumor model was significantly reduced (~16% compared to day 1 levels) on day 11 after HBO treatment (2 bar, pO_2 = 2 bar, 4 exposures à 90 min), whereas control tumors increased almost 100 % in volume during the same experimental period. Significant decreases in tumor cell proliferation, tumor blood vessels and collagen fibrils, together with an increase in cell death, are consistent with tumor growth reduction and tumor stroma influence after HBO treatment. Gene expression profiling showed that HBO induced a mesenchymal-to-epithelial transition (MET) with coordinated expression of gene modules involved in cell junctions and attachments together with a shift towards non-tumorigenic metabolism.

3.2 Paper II

Hyperoxia increases the uptake of 5-fluorouracil in mammary tumors independently of changes in interstitial fluid pressure and tumor stroma.

Uptake of [H^3]-5-fluorouracil in the DMBA-induced tumors were increased with more than 50% immediately after a single HBO treatment (2 bar, pO_2 = 2 bar, 1 exposure of 90 min). However, this effect was not found when measured 24 hours after the last repeated HBO treatment (2 bar, pO_2 = 2 bar, 4 exposures à 90 min). Tumor interstitial fluid pressure (P_{if}), lymphatic structures and collagen content decreased significantly after HBO. However, HBO did not induce any change in

oxygen stress (measured as malondialdehyde levels) and transcapillary transport in the tumors.

3.3 Paper III

Tumor-stroma interactions in 4T1 mammary tumors with and without enhanced oxygenation.

A new tumor model with dsRed expressing 4T1 mammary tumors in eGFP (enhanced green fluorescent protein) expressing NOD/SCID (Non-obese diabetic/ Severe combined immunodeficiency) mice was successfully established. Fluorescence microscopy, confirmed a successful separation of the tumor- and stromal cells after Fluorescence Activated Cell Sorting. Gene expression profiling demonstrated that highly up-regulated genes in the untreated tumor stroma included constituents of the extracellular matrix and matrix metalloproteinases. Furthermore, tumor growth was significantly inhibited by HBO. However, it did not inhibit metastasis over time. Immunohistochemistry and gene expression data showed a significant anti-angiogenic effect after intermittent HBO (2.5bar, 100% O_2, 3 exposures à 90 min), whilst daily HBO (2.5bar, 100% O_2, 7 exposures à 90 min) did not show the same response. Neither morphology, proliferation nor the amount of cell death was significantly changed after HBO.

4. Methodological Considerations

Detailed descriptions of methods are provided in the respective papers included in this thesis. However, aspects influencing choice of animal models and treatment, together with their advantages and limitations, are discussed in this section.

4.1 Animal Models

We used two different mammary tumor models in this thesis. The first two papers were performed on chemically-induced (DMBA) tumors in rats. In Paper III, 4T1 mammary tumors in mice were used.

4.1.1 Chemically-Induced DMBA Adenocarcinomas

Dr. Charles Brenton Huggins developed the DMBA-induced rat mammary carcinoma model in 1961 [64]. The polycyclic aromatic hydrocarbon DMBA functions as a immunosuppressor, in addition of being a potent organ-specific carcinogen, most commonly inducing skin and mammary tumors in animals [65]. This model is now one of the most extensively investigated laboratory animal models mimicking human breast cancer. Histopathological and immunohistochemical characterization confirm that this is a good model for the changes occurring early in the multistep process of mammary gland carcinogenesis [66]. Investigation on UHKBR-01, a cell line derived from DMBA-induced rat mammary tumors, revealed that the cells are oestrogen- and progesterone-receptor positive, like other established breast cancer cell lines such as MDA-MB-231 and MCF7, which both are derived from human tumors [65]. Taken together with the fact that the effect of HBO on tumor growth, alone and in combination with 5FU, already was tested and proven to work reliably in this tumor model [61, 63], made it a natural choice for Paper I and II.

4.1.2 Murine 4T1 Mammary Carcinomas

4T1 is one of four cell lines isolated from a spontaneously arising autochthonous BALB/cfC3H mammary tumor [67]. Normally, human xenograft models metastasize poorly or to unexpected sites in mice. In contrast, murine tumor models often have more similar metastatic characteristics to that observed in cancer patients. Thus, murine 4T1 cells in mice metastasize to lung, liver, brain and bone through the hematogenous route, all sites affected in human breast cancer [68]. In this thesis, 4T1 cells were implanted in NOD/SCID mice [69]. This stock of mice has been shown to have multiple defects in adaptive and innate immune function, and allow a high percentage of tumor take, and thereby represent an advanced model for xeno-engraftment studies [70]. Further, most of the experiments in Paper III are performed on dsRed transfected 4T1 tumor cells injected into eGFP expressing NOD/SCID mice. This tumor model enables us to visualize in detail the co-localization of the tumor and host cells, in addition to enabling separation of the two compartments using FACS (fluorescence activated cell sorting). This gives us the opportunity to investigate changes occurring in the tumor cells and the stromal cells separately, and thereby having a better chance of finding possible mechanisms involved in the effect HBO has on tumors. Niclou *et al.* [70] showed that the eGFP expressing NOD/SCID stock had an immunological profile comparable to the non-transgenic parental line, and that we can therefore compare results from the NOD/SCID model with the fluorescent stock. Coincident with tumor cell implantation, a 17β-estadiol pellet (0.18mg/pellet-60 day release) was implanted into the neck of the mice. 4T1 lack estrogen receptor α (ERα), and is therefore non-responsive to estrogen stimulation of growth. Nevertheless, Banka *et al.*[71] found that estrogen stimulation can affect the metastatic capability of 4T1 tumors. Thus, we chose to implant estrogen-pellets to be able to investigate tumor metastasis in addition to tumor growth.

4.1.3 Change of Animal Model

As Stuhr *et al.* [61] and Raa *et al.* [63] both achieved promising results with HBO on the DMBA-induced mammary tumor model in rats, we wished to follow-up these studies to thoroughly elucidate how enhanced oxygenation influence tumor progression and response to chemotherapy in this model. Thus, both Paper I and II include studies on the DMBA-induced tumor model. Furthermore, in Paper III we wanted to test the hypothesis of enhanced oxygenation in a different mammary tumor model, to see if HBO had a general tumor-inhibitory effect on mammary tumors. Development of a mammary tumor model with dsRed 4T1 tumor cells injected in eGFP mice, enabled us to separately investigate oxygen-related changes occurring both in the tumor cells and in the tumor stroma. As of today, eGFP rats are not available, but if so, rats would be preferred, as tumor growth studies in rats enables longer experimental periods without influencing with the mobility and welfare of the animals, and also provides more tissue for analysis.

4.2 Hyperbaric Oxygen

In clinical practice, side effects of HBO are rare and mild because of the relatively low oxygen pressures used and the limited time of the treatment sessions.

4.2.1 Side Effects due to changes in Atmospheric Pressure

During compression and decompression of the hyperbaric chamber, the pressure-volume relationship described in Boyles law (Formula 4), may cause unpleasant or more serious side effects.

The most frequent problem caused by changes in atmospheric pressure is middle ear barotraumas, due to insufficient equilibration of the middle ear. Further, perforation of the oval window, and thereby leakage of perilymph from the inner ear to the middle ear, is a rare, but serious side effect. The most serious effect of barotrauma is pneumothorax, wherein air or gas is present in the pleural cavity, and leads to

collapse of the lung. However, this state has only been reported in a few cases, and always in comatose or ventilated patients [54]. Most of the adverse effects of hyperbaric conditions are, however, related to rapid decompression, i.e. relieving pressure [72]. Normally, gases of the atmosphere are in solution in the body tissues. Exposure to increased environmental pressure, leads to increased solubility of these gases in the body fluids, until saturation is reached. A sudden decrease of environmental pressure will lead to bubble formation. Still, the bubbles are mainly caused by nitrogen, as the body quickly can consume and deal with excess oxygen and carbon dioxide, and these bubbles induce decompression sickness. Decompression sickness can take several forms. Type I, or “the bends”, is related to pain in the major joints. Type II is a more serious condition and involves pulmonary, cardiac, ophthalmic, or neurological symptoms and the possibility of shock or coma [72]. HBO have shown beneficial effects on patients with decompression sickness, by reducing the bubble size, increasing the dissolution of the gas bubbles and counteract the hypoxic condition [56].

In the present study, both compression and decompression of the chamber was performed following a conservative schedule, using 3-5 min to reach the desired pressure and ~10 min to decompress the animals. The animals did not show any sign of discomfort during pressure changes.

4.2.2 Side Effects due to Oxygen Partial Pressue

In 1878, Paul Bert was the first to demonstrate the toxicity of oxygen. Thus, seizures resulting from acute oxygen toxicity to the central nervous system are still referred to as the "Paul Bert effect". Now it is clear that brain oxygen toxicity is pressure dependent and the immediate toxicity is reached by breathing 100% oxygen at 4-5 bar [73]. At lower pressure the threshold is time dependent. Later, Lorrain Smith discovered that even moderate oxygen tensions could lead to serious and fatal lung injuries when the exposure time was long enough. This chronic pulmonary oxygen poisoning is therefore called the “Lorrain Smith effect”. Oxygen lung toxicity is

related to the cumulative damage from oxygen free radicals to lung parenchyma and airways and may thereby only occur during prolonged hyperbaric treatments [54]. Further, symptoms of oxygen toxicity may include disorientation, breathing problems and vision changes. Repeated HBO sessions may lead to transient myopia [74], but is reversed a few weeks after the cessation of treatment. In any environment with raised oxygen pressure, there is an increased risk of fire.

Pott *et al.* [75] found no persistent effect on pulmonary function after 90 min HBO treatment of 2.4 bar. Together with the study by Lambertsen *et al.* [73], this clearly show that the pressure and time-frame used in our experiments are within safe range and that neither acute nor chronic oxygen toxicity was considered a problem. Furthermore, to avoid the fire hazard in the pure O_2 environment, the animals were showered lightly with water prior to entering the pressure chamber. Additionally, the chamber was litter free, oil free and all electrical connections were disconnected.

4.2.3 Change of HBO Protocols

In paper I and II, the animals were treated 4 times for 90 min in a 2 bar and 100% O_2 atmosphere. This protocol was chosen, as these studies were performed as follow-up studies of Stuhr *et al.* [61]. They found that an extended experimental period, with 7 HBO treatments over 23 days, gave no further reduction in tumor size, and thus 4 treatments were chosen [61]. Additionally, Paper II included a single HBO treated group, to be able to study the acute effect of increased pO_2.

Raa *et al.* [63] studied the effect of normobaric- and hyperbaric hyperoxia. This study, combined with the study by Stuhr *et al.* [61] indicate that the growth reduction is dose-dependent on pO_2. Therefore, in Paper III, the HBO protocol was slightly changed. First, the atmospheric pressure was increased to 2.5 bar, to see if increased pO_2 would induce an even greater growth-inhibitory response. Furthermore, due to the change of animal model from rats to mice and the aggressiveness of the 4T1 tumors, the control mice could not carry the tumors for more than 8 days. Thus, the

treatment protocol was reduced from 4 treatments over 11 days, to 3 treatments over 8 days. Additionally, one group of mice was treated daily with HBO, to elucidate if this could possibly potentiate the inhibitory effect of HBO, or if the tumors would adapt to the enhanced oxygenation, and thus display less response to the treatment.

5. General Discussion

Although cancer research has made significant progress, important questions within this field are still unanswered. Hypoxia is one of the known features of solid tumors, and has been connected to several important parameters influencing both tumor growth and response to therapy. Thus, the rationale for focusing on tumor oxygenation in this thesis, is the hypothesis that hyperoxia, “the flip of the coin”, might counteract some of the mentioned negative aspects of hypoxia and thereby lead to additional insight into the effects of tumor oxygenation.

5.1 HBO in Cancer Treatment

As oxygen is believed to be required for all the major processes of wound healing, including resistance to infection, collagen deposition, angiogenesis and epithelisation [76], one feared that HBO would have the same proliferative effect in tumors. Therefore, throughout the 1950ies and 1960ies, the effect of HBO on cancers was tested to elucidate if HBO did promote tumor growth. The results from the studies were reviewed by both Feldmeier *et al.* [59] and Daruwalla *et al*. [60]. The reviewed studies included different types of cancers, with and without additional therapy, and thus had a variety of responses. Nevertheless, both reviewers concluded that HBO did not promote tumor growth, and that the use of HBO in patients with malignancies was considered safe. Subsequently, systematic research on HBO and cancer was terminated. It is important to emphasize that an effect of HBO treatment *per se* on tumors possibly depends on multiple factors, including tumor type and stage, as well as the timing, duration, atmospheric pressure and number of HBO exposures. *In vitro* studies have shown that there are discrepancies in growth fraction between cell lines exposed to hyperoxia [77], indicating differences in response to oxygen between different tumor types. This is reflected in the literature where patients with head and neck cancers tend to be most responsive to HBO therapy and patients with cervical and bladder cancer least responsive [60]. Thus, the observed variety in response to

HBO observed by Feldmeier *et al.* [59] and Daruwalla *et al.* [60] can therefore be ascribed to both differences in types of tumors, but also to the large variety in HBO treatment protocols. Nevertheless, studies from our group have demonstrated a growth-inhibitory effect of HBO on mammary tumors [61, 63], and therefore elucidation of HBO's effect on mammary tumors were the scope of this thesis. Previous studies performed on HBO and cancers have only included evaluation of tumor growth and/or metastasis. Hence, the work performed in this thesis have looked further into the changes oxygenation causes in the tumor tissue, and considered the possibility that HBO might have been too early abandoned as a possible cancer treatment, at least as a treatment adjuvant.

Hyperoxia and the Angiogenic Switch

Even though clinical experience has shown that hyperoxia stimulate healing of wounds, the mechanisms accounting for the dependency of oxygen on angiogenesis, are largely unknown [78]. In contrast to the comprehension that oxygen leads to angiogenesis and collagen deposition in non-tumor tissue, our work show that HBO has an anti-angiogenic effect and leads to less fibrosis in tumors [62, 63, 79, 80]. Thus, this thesis contributes to new understanding of oxygen and its influence on angiogenesis in tumor tissue. It is well known that the HIF-1α-pathway is activated during hypoxia. It is therefore paradoxical that increased oxygenation should lead to increased angiogenesis in wound healing of non-tumor tissue, as HIF-1α is degraded when oxygenated. On the other hand, the anti-angiogenic effect we observed after hyperoxia, where several growth factors known to be induced by HIF were significantly reduced, supports the fact that HIF-1α is degraded during oxygenation. Based on the fact that angiogenesis creates new blood vessels that can nurture the tumor tissue, anti-angiogenesis could help explain the tumor-inhibitory effect. However, since both daily and intermittent HBO treatment of the 4T1 tumors in Paper III induced a growth-inhibitory effect, but only the intermittent group displayed anti-angiogenesis, angiogenesis cannot be the main determining factor behind the observed growth inhibition.

Hyperoxia and Cell Survival

Together, Papers I and III indicate that HBO has a general tumor-inhibitory effect on mammary tumors. However, the extent of tumor-inhibition is far greater in the chemically-induced DMBA-tumors than in the 4T1 tumors. This also shows that despite the increased atmospheric pressure (from 2 to 2.5 bar); used in treating the 4T1 tumors, the HBO treatment did not induce a more pronounced tumor-inhibitory response. This indicates that the previously observed dose-dependency of pO_2 in the DMBA tumors [63] is not applicable when changing tumor models. Immunohistochemistry revealed differences in response to HBO with respect to both cellular proliferation and cell death in the two tumor models. While HBO display an anti-proliferative and pro-apoptotic effect in the DMBA-tumors, no significant changes in these parameters were observed in the 4T1 model. One might speculate if the 4T1 tumor were less hypoxic, and thereby displayed less response to enhanced oxygenation.

Hyperoxia and the Glycolytic Switch

Lòpez-Làzaro [81] published a review in 2009 questioning the importance of hypoxia in tumor progression, and proposed the alternative idea, viewing cancer as a process in which oxygen metabolism is altered from an energy-generating pathway to an ROS-producing pathway. With this in mind, the results from Paper I indicate a switch to a non-tumorigenic metabolism, while this metabolic-switch was not observed in the 4T1 tumors in Paper III. This discrepancy in metabolism could be a reasonable explanation for the difference in the extent of tumor growth-inhibition in the two mammary tumor models.

Hyperoxia and Metastasis

Metastasis is the major cause of cancer deaths [40]. As stated in the introduction, hypoxia increases the metastatic capability of tumors [40, 41]. In accordance to this, results from Paper I indicated that oxygen is an important factor in the “switch” from EMT to MET, leading to less aggressive and invasive tumors. On the other hand, the

supplementary results in Paper III show that HBO did not seem to inhibit the metastatic capability of the 4T1 tumor model over time. Thus, oxygen does not induce the same "switch" in all tumor models. Due to the fact that the DMBA tumor is not a metastatic model, the influence of MET on the metastatic behaviour is hard to determine. However, the significance of O_2 as a factor in the induction of MET in DMBA-induced tumors is still interesting, since such a transition is believed to induce a less aggressive phenotype.

Only a few other experimental studies have elucidated the effect of HBO *per se* on breast cancer growth and metastasis. Granowitz *et al.* [82] and Chen *et al.* [83] concluded that HBO had a significant inhibitory effect on mammary cell proliferation *in vitro*, which is in accordance with the results from our *in vivo* models. Further, McCredie *et al.*[84] and Haroon *et al.* [85] found that HBO did not promote metastatic capability. Nevertheless, even though HBO display tumor-inhibitory effects in the mammary tumor models described in this thesis, there are too few studies supporting HBO inhibitory effects as a stand-alone mammary cancer therapy. However, combining enhanced oxygenation with chemotherapy, or another tumor-inhibiting modality, may be one way to synergistically improve the treatment effect.

5.2 HBO as a Chemoterapeutic Adjuvant

As opposed to studying the effect of HBO *per se* on tumor growth, several studies have elucidated HBO as an adjuvant to conventional chemotherapy, both experimentally and in a clinical setting [61, 82, 86-92]. The studies were performed on different tumor types, with different chemotherapeutic drugs, and thus with great variation in response. Teicher [50] underlines that the importance of hypoxia on the response to chemotherapy is highly drug dependent. As stated in the introduction, hypoxia-mediated chemo-resistance has been ascribed to: 1) Some drugs and radiation requiring oxygen to generate ROS to be maximally cytotoxic. 2) Altered

cellular metabolism reducing drug cytotoxicity. 3) Hypoxia leading to genetic instability, which can lead to more rapid development of drug-resistant cells [50]. In addition the cytotoxicity, availability of the chemotherapeutic drug is important to obtain maximal effect. Tumor tissue anatomy influence transport of intravenously injected substances to the tumor cells, and thus the efficacy of the drug.

5FU is a chemotherapeutic drug widely used in treatment of cancers, including colorectal cancer and breast cancers [93]. Paper II showed that the uptake of radioactively labelled 5FU was increased compared to control immediately after one HBO treatment, when the pO_2 was still high, but not 24 hours after the last of 4 repeated treatments. Oxygen-related changes observed in the tumor stroma, like collagen density and P_{if}, did not seem to influence the uptake, as thoroughly discussed in Paper II. Rump *et al.* [94] speculated if hyperbaric or hyperoxic conditions may affect the drug distribution by changing the catalytic activity of drug metabolizing enzymes, the hemodynamics and membrane permeability. The synergistic effect of HBO and 5FU observed by Stuhr *et al.* [61] is therefore not only ascribed to increased cytotoxicity, but seems to be related to increased availability of the chemotherapeutic drug, due to increased uptake.

Pre-clinical studies have shown, that combining HBO with the right chemotherapeutic drug, might improve the treatment outcome of several types of cancer [61, 82, 88-92, 95]. However, further research within this field is needed to elucidate the mechanisms underlying the synergistic effect of oxygen and chemotherapy.

6. Conclusions

Our overall aim in this thesis was to study the effect of enhanced oxygenation on tumor growth, progression and response to therapy.

We therefore addressed and answered the following specific aims:

1. **To investigate the effect of HBO therapy on tumor growth and progression in two different mammary tumor models, by elucidating cell proliferation, cell death and angiogenesis.**

   HBO significantly inhibited mammary tumor growth in both the chemically-induced DMBA tumors and the 4T1 tumors. Intermittent/Repeated HBO had a significant anti-angiogenic effect in both tumor models. However, while HBO had a pro-apoptotic and anti-proliferative effect on the DMBA-tumors, no such effect was apparent in the 4T1 tumors. **(Paper I and III)**.

2. **To study the gene expression profile of both untreated and HBO treated tumors.**

   Gene expression profiling showed that HBO induced a Mesenchymal-to-Epithelial Transition and a shift towards a non-tumorigenic metabolism in the DMBA-induced mammary tumors, leading to more differentiated and less aggressive tumor phenotype**.** No such transition was observed in the 4T1 tumors. Additionally, the immunohistochemical findings on angiogenesis, cell proliferation and cell death were supported by results from the gene expression analysis **(Paper I and III).**

3. **To develop a mammary tumor model, to be able to study both aim 1 and 2, in addition to elucidate tumor-stroma interactions.**

   A new mammary tumor model was established, with dsRed transfected 4T1 tumor cells injected into eGFP expressing NOD/SCID mice. This mammary tumor model enabled us to separate tumor and stromal cells, and demonstrated that the two compartments are characterized by distinct gene expressions, both in the native state and following HBO treatments **(Paper III).**

4. **To elucidate the effect of HBO on the uptake of a conventional chemotherapeutic drug, 5FU, into the tumor tissue, and study possible oxygen-related changes occurring in the tumor stroma that might have influenced the chemotherapeutic response, as interstitial fluid pressure, amount of collagen, fluid distribution and reactive oxygen species.**

   HBO increased the uptake of [H^3]-5FU in the DMBA induced mammary tumors *per se*, independently of changes in P_{if}, oxygen stress, collagen fibril density or transendothelial transport alone **(Paper II).**

7. Future Perspectives and Concluding Remarks

Paper I, II and II have taken us one step closer to determining the effect of enhanced oxygenation on mammary tumor growth, progression and response to therapy by elucidating changes occurring in the tumor tissue.

Due to the observed tumor-inhibitory effect of HBO on both the DMBA- induced- and the 4T1 mammary tumors, we aim to study a human mammary tumor model, MDA-231, *in vivo*. By elucidating the response to this human tumor model, we wish to increase the clinical relevance of the studies and get an even closer look into the background for the response to HBO.

To be able to reach our ultimate goal of finding the mechanisms by which oxygen influence tumor development and response to therapy, further *in vitro* studies have been outlined. We aim to study cell proliferation of the breast cancer lines, 4T1, MDA-MB-231 and MCF-7 *in vitro*, after exposing the cells to three different oxygen-environments, hypoxia (2% O_2), normoxia (20% O_2) and hyperoxia (80% O_2). After incubation of the cells in the different oxygen-concentrations, gene expression studies will hopefully help us uncover possible oxygen-sensitive genes involved in tumor growth and progression. Thus, by comparing genes that respond in opposite direction after hypoxic and hyperoxic conditions, we can possibly indentify important oxygen-dependent genes for further evaluation.

Ultimately, we wish to study Lentiviral/siRNA over-expression and knock-out strategies of the possible oxygen-dependent genes. This will first be performed on cell lines, before prospective functional validation *in vivo*.

In turn, this may provide important new information as to how growth of cancers might be controlled, to potentially provide new treatment strategies for both breast cancer, but also other types of neoplasms.

Errata

Introduction:

1.3, line 12, Michieli et al. should read Michieli.

1.3.5, line 1, Grey et al. should read Gray et al.

Paper I:

Table 3, Agilent Human Whole Genome Oligo Microarrays should read Agilent G413F Whole Rat Genome (4x44k) Oligo Microarray Kit.

Figure 3C, should not contain heading (Number of collagen fibrils (2C)).

Paper III:

Discussion, page 13, paragraph 3, line 3, fgf should read FGF.

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Appendix

Table 3: Pressure conversion table.

To From	atm	bar	kPa	msw
atm	1	1.013	101.325	10.123
bar	0.987	1	100	10
kPa	9.869×10^{-3}	0.010	1	0.1
msw	0.099	0.1	10	1

Table 4: Studies on the effect of hyperbaric oxygen and malignancy, both alone and in combination with conventional treatment, from 2004-2010. ↔ indicates no effect on growth, ↓indicates decreased growth. If two symbols are given, the effect is mixed. Exp=exposure, adm=administration.

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Chong *et al.* [96]	2004	*In vivo*	Human prostate (LNCaP) cells in immunodeficient mice.	0.236MPa, 20 exp. à 90 min, 5 days a week for 4 weeks.		HBO does not accelerate the growth of indolent prostate cancer.	↔
Stuhr *et al.* [61]	2004	*In vivo*	DMBA-induced mammary tumors in rats.	2 bar, 4 exp. à 90 min, 11 days. 7 exp., 23 days.	5FU	HBO significantly reduced tumor size and increased the effect of 5FU.	↓
Sun *et al.* [97]	2004	*In vivo*	Human oral cancer cell line in mice.	2.5 atm, 20 exp. à 90 min		HBO did not promote the growth and proliferation.	↔
Shi *et al.* [98]	2005	*In vivo*	Head and neck squamous cell carcinoma (Sq20B and Detroit 562) in mice.	2.4 atm, 90 min 5 times a week for 2-4 weeks.	Radiotherapy	HBO had no effect on tumor growth, neither alone nor in combination with radiation. However, HBO did reduce hypoxia.	↔

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Hjelde *et al.* [99]	2005	*In vitro*	Traditional cell carcinoma (AY-27), Human primary colon- adenocarcinoma (WiDr) and human colon-adenocarcinoma cell line (SW480).	100, 200, 300, 400kPa O_2 for 30 min.	Photodynamic therapy	The decrease in cell survival was not influenced by hyperoxia.	↔
Granowitz *et al.* [82]	2005	*In vitro*	Mammary cells from normal epithelia, primary tumor and metastatic tumor + human MCF7 cell line.	2.4 atm	Melphalan, Gemcitabine and Paclitacel.	HBO inhibits cell proliferation alone, and augmented the effect of all three chemotherapeutic agents.	↓
Heys *et al.* [87]	2006	Clinical	Locally advanced breast carcinoma.	2.4/2.0 atm, daily exp. for 10 days.	Cyclo-phosphamide ,Doxorubicin ,Vincristine.	HBO lead to reduced oedema, but showed no reduction in tumor volume or increase in vascularisation.	↔
Daruwalla *et al.* [100]	2006	*In vivo*	Dimethyl hydrazine (DMH) induced primary colon carcinoma cell line in mice.	2.4 atm, 90 min daily exp. for 7, 13, 19 and 25 days.		HBO caused no reduction in liver metastasis. HBO-treated tumors were generally smaller than the controls.	↔

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Daruwalla *et al.* [101]	2007	*In vivo*	Primary colon carcinoma cell line in mice.	2.4 atm, 5 times à 90 min over 9 days.	SMA-pirarubicin	HBO therapy alone gave no effects, however in combination with SMA-pirarubicin HBO gave reduction in both liver metastasis and tumor growth, and showed increased levels of necrosis.	↓
Raa *et al.* [63]	2007	*In vivo*	DMBA-induced mammary tumors in rats	Hyperoxia (100% O_2) or 1.5 bar, 4 exp. à 90 min over 11 days.	5FU	Both hyperoxia and HBO induced tumor growth retardation, in addition to reducing vascular density and enhancing cell death.	↓
Haroon *et al.* [85]	2007	*In vivo*	Mouse mammary adenocarcinoma 4T1-GFP cell line in nu/nu mice.	2.8 ATA for 45 min daily (5/ week) up to 5 weeks.		The total metastatic load was reduced after HBO treatment.	↓
Stuhr *et al.* [62]	2007	*In vivo*	BT4C rat glioma xenografts in nude rats.	100% O2 or 2 bar HBO, 3 exp. à 90 min over 8 days.		Normobaric and hyperbaric hyperoxia inhibited tumor growth, induced cell death and reduced the vascular density.	↓

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Chen *et al.* [83]	2007	*In vitro*	Human leukemia (Jurkat), multiple myeloma (NCl-H929), carcinoma (A549) and breast adenocarcinoma (MCF-7) cell lines.	2.5 or 3.5 ATA oxygen or air for 2-12 h.		HBO induced apoptosis in the hemapoietic Jurkat and NCl-H929 cells, but not in A549 or MCF7 cells. HBO did also inhibit cell proliferation in the MCF7 cells.	↓/↔
Schönmeyr *et al.* [102]	2008	*In vitro and in vivo*	Murine squamous cell carcinoma (SCC-VII) cell line in vitro and in mice.	2.1atm 8 daily exp. à 90 min.		HBO does not alter cell growth and proliferation neither *in vivo* nor *in vitro.*	↔
Ohguri *et al.* [86]	2009	Clinical	Patients with non-small-cell lung cancer (NSCLC).	Weekly chemotherapy and hyperthermia was adm. immediately after HBO.	Paclitaxel and carboplatin.	75% of patients with HBO had an objective response. Hyperthermia and HBO may be a feasible and promising modality for treating NSCLC.	↔/↓
Suzuki *et al.* [88]	2009	Clinical	Patients with recurrent malignant or brainstem gliomas.	0.2MPa, 60 min during i.v. adm. of carboplatin + 24 h after drug adm.	Carboplatin.	The results suggest that HBO therapy prolongs the biological residence time of carboplatin.	↓

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Kawasoe *et al.* [89]	2009	*In vitro and in vivo.*	Mouse osteosarcoma (LM8) cell line in vitro and implanted in mice.	2.5 atm for 90 min.	Carboplatin.	HBO markedly suppressed the growth and metastasis both in vitro and in vivo. HBO also reinforced the effect of carboplatin.	↓
Moen *et al.* [79]	2009	*In vivo*	DMBA-induced mammary tumors in rats.	2.0 bar, 4 exp. à 90min, 11 days.		HBO inhibited tumor growth, and showed anti-angiogenic, anti-proliferative and pro-apoptotic effect, and more differentiated and less aggressive tumors.	↓
Moen *et al.* [80]	2009	*In vivo*	DMBA-induced mammary tumors in rats.	2.0 bar, 4 exp. à 90min over 11 days or 1 exp. à 90 min.	5FU	HBO increased the uptake of [H^3]-5FU per se.	↓
Tang *et al.* [103]	2009	*In vivo*	Human prostate PC-3 cells in immunodeficient mice.	2atm, 20 exp. à 90 min, 5 days a week for 4 weeks.		HBO did not have any tumor stimulatory effects.	↔

Study	Year	Type of study	Tumor type	HBO protocol	Additional therapy	Comment	Outcome
Selvendiran *et al.* [104]	2010	*In vivo*	Human ovarian cancer xenograft.	2 atm, 90 min daily for up to 21 days.	Cisplatin	HBO induced a significant reduction in tumor volume, both alone and in combination with cisplatin.	↓
Ohgami *et al.* [105]	2010	*In vitro*	Molt-4 human leukemia cells.	3.5 atm, 90 min.	Artemisinin	Combined artemisinin and HBO decreased cell growth.	↓
Peng *et al.* [91]	2010	*In vitro*	Nasopharyngeal carcinoma CNE2Z cells.	2 ATA, 85% O2, exp. à 90 min repeated at a 4 h interval.	5FU	HBO treatment inhibited proliferation, but not metastasis. A synergistic effect in combination with 5FU was only observed after 48 h.	↓/↔