Intracranial meningiomas, the VEGF-A pathway, and peritumoral brain edema

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1. PAPERS INCLUDED IN THE THESIS

The thesis is based on two published first authorship (paper I and III), and one first authorship manuscripts. Paper II is a methodological study of a novel method for mRNA quantification, while papers I and III address the VEGF-A pathway and PTBE in meningiomas.

PAPER I
Nassehi D, Dyrbye H, Andresen M, Thomsen C, Juhler M, Laursen H, Broholm H. Vascular endothelial growth factor A protein level and gene expression in intracranial meningiomas with brain edema. APMIS. 2011 Dec;119(12):831-43.

PAPER II
Nassehi D, Dyrbye H, Sørensen LP, Juhler M, Laursen H, Broholm H. VEGF-A mRNA measurement in meningiomas using a new simplified approach: Branched DNA and chemiluminescence. (Submitted)

PAPER III
Nassehi D, Sørensen LP, Dyrbye H, Thomsen C, Juhler M, Laursen H, Broholm H. Peritumoral brain edema in angiomatous supratentorial meningiomas: An investigation of the VEGF-A pathway. APMIS. 2013 Feb (early view online). DOI: 10.1111/apm.12052.

2. THEORETICAL SECTION

MENINGIOMAS
Meningiomas are some of the most common tumors of the nervous system. They are derived from the arachnoidea cells and can be either intracranial (90%) or spinal (1). Although mostly benign, meningiomas can cause serious morbidity and mortality based on their localization (2).

Epidemiology
The meningioma prevalence was 97.5/100.000 in the U.S.A. in 2009–2010, while the incidence rates were 3.6 and 8.4 for males and females respectively (2). In Scandinavia the incidence rates were 1.4–1.9 and 2.6–4.5 for male and female patients respectively in 1995–1997 (3). The meningioma incidence rate increases with age, and so meningiomas are rare in children, except in cases with neurofibromatosis type II or secondary meningiomas after radiotherapy. The high female:male ratio (2:1) is less obvious in WHO grade II or III meningiomas, meningiomas in children, secondary and spinal meningiomas.

Risk factors
Apart from age and gender, several risk factors have been identified. Radiation, both therapeutic and accidental, has been linked to the incidence of secondary meningiomas (4-7). These patients usually developed singular or multiple meningiomas 20 to 40 years after exposure.

The autosomal dominant disorder, neurofibromatosis type II, significantly increases the risk of schwannomas and meningiomas. Half of the individuals with the disorder have meningiomas, and in many cases multiple meningiomas are present (8). The disorder is due to a mutation in the tumor-suppressor NF2-gene on chromosome 22.

It is possible than hormone exposure, e.g. during pregnancy, with the use of contraceptive medications or hormone replacement therapy, may increase the risk of meningiomas due to the high expression of progesterone receptors on meningioma cell membranes (9). Also, women with breast cancer have a slightly increased risk of meningioma. This could be linked to the high expression of sex-hormone receptors in both tumors.

Head trauma was previously believed to increase the risk of intracranial meningiomas, but the evidence is not strong enough to give substance to this hypothesis (10).

Cell phone use has not been linked to increased incidence in meningiomas in a large danish study (11), although some critics argue that the study was conducted too early and that an increase in meningioma incidence will not present itself in advance of 10–20 years.

Pathology
Meningiomas are classified as grade I, II, or III based on the WHO 2007 classification guide lines (12). Grade I tumors are benign, grade II tumors have increased mitotic activity, and grade III tumors are malignant. Furthermore, grade II and III meningiomas

have an increased risk of recurrence. Although only grade I tumors, both angiomatous and secretory meningiomas are known for prompting PTBE formation (12). Angiomatous meningiomas are also known for their increased capillary length. Because of the embryological relationship between arachnoidea cells and cells of the uterus, both share the presence of female sex-hormone receptors on their surface.

Studies report that WHO grade I meningiomas account for more than 90% of all meningiomas, while grad II and III meningiomas account for 5–7% and 1–3% respectively (1).

Clinical presentation
Because of their development from the meninges and their benign nature, meningiomas very rarely grow invasively. Meningiomas often present themselves with symptoms depending on their size, growth rate and localization. Epileptic seizures, visual changes, hearing loss, paresis, changes in mental state, and obstructive hydrocephalus are all known symptoms of intracranial meningiomas. Most (90%) of all intracranial meningiomas are supratentorial (1), and more than 50% of all meningiomas develop peritumoral brain edema (PTBE), which can aggravate the symptoms even further (13). Due to a very slow growth rate, some meningiomas remain asymptomatic and are only diagnosed accidentally during radiological investigation for other illnesses or during autopsy.

Diagnosis
Because of the characteristic presentation and localization of meningiomas, they can often be diagnosed based on computed tomography (CT) or magnetic resonance imaging (MRI) alone.

MRI is the preferred modality for diagnosis. The origin of the meningioma can often be visualized as well as the size and extension of the PTBE, if such is present. Meningiomas are iso- or hypodense on T1-weighed images and iso- or hyperdense on T2-weighed images. They are presented as well-defined extra-axial masses that displace the brain. They can be diagnosed on non-contrasted CT, although contrasted images are preferred because the tumor boundaries are better visualized and the PTBE is more easily diagnosed. Definite diagnosis is based on histological findings after surgery. Meningiomas are readily recognizable due to their homogenous appearance and the presence of whorls. They can usually be diagnosed on haematoxylin-eosin (HE) histological sections alone.

Treatment
Meningiomas are treated differently based on their presenting symptoms. Because meningiomas can normally be diagnosed based solely on MRI, a wait and see approach with routinely scheduled MRI controls is often chosen when dealing with asymptomatic patients. However, in the case of symptomatic patients, surgery is often the ultimate treatment. Usually, surgery can be scheduled well into the future, because of the slow progression of meningiomas, although some patients can suddenly present themselves with massive PTBE and risk of incarceration of the brain stem or herniation of the cerebrum. Some patients receive either radiation therapy or stereotactic radio-surgery which reduce the tumor size (14). These treatments can be complicated by increased intracranial pressure due to secondary brain edema.

In patients with PTBE, intravenous or oral steroid hormone therapy is administered in order to reduce the PTBE. Naturally, the cause of edema generation is eliminated with tumor removal, but in most cases pre-surgical elimination or reduction of edema is desirable in order to reduce surgical morbidity. Still, steroid therapy is not an ideal choice because its effects are non-specific, it is not always effective, and has long-term side effects (15).

PERITUMORAL BRAIN EDEMA
Classically, brain edema is classified as either cytotoxic or vasogenic. Cytotoxic brain edema develops when the metabolism of the brain cells is compromised, typically due to early stroke, or other factors that result in ischemia. The cellular sodium-potassium pump fails due to the impaired metabolism, resulting in retention of intracellular sodium and water.

Vasogenic brain edema develops as the result of a compromised blood-brain barrier, typically due to trauma, hemorrhage, invasive tumor growth, and late stages of cerebral ischemia. When blood or plasma is introduced to the extracellular matrix of the CNS, the fluids spread along the axon sheets of the brain, eventually reaching the grey matter. The influx of proteins also increases the osmolarity of the extracellular matrix, resulting in an osmotic flow gradient which further expands the extracellular matrix. Although brain edema is traditionally classified as either cytotoxic or vasogenic, a combination of both mechanisms is often responsible for the edema formation in reality.

Under normal conditions, the blood-brain-barrier is a highly selective membrane between the brain and the blood. It consists of non-fenestrated capillaries bound together by transmembrane and cytoplasmatic proteins, e.g. claudin, occludin, ZO-1, ZO-2, ZO-3, and cadherin to name a few (16). Presently it is hypothesized that either a decreased expression of functioning tight-junctions or a disruption of normally expressed tight-junction proteins results in the compromised blood-brain-barrier and the formation of PTBE (15).

VASCULAR ENDOTHELIAL GROWTH FACTOR A
Vascular endothelial growth factor A (VEGF-A), also known as Vascular Permeability Factor or VEGF165, is a potent mitogenic and angiogenic disulfide-linked homodimer responsible for angiogenesis in several organs including the CNS. VEGF-A has been shown to increase extravasation of proteins from tumor associated capillaries, thus the monicker Vascular Permeability Factor (17). VEGF-A is part of the VEGF family of proteins which includes VEGF-B, VEGF-C, VEGF-D, and placental growth factor.

VEGF-A expression is increased by reduced blood flow and ischemia, and is involved in the growth and expansion of tumors in a cycle where tumor growth results in ischemia, which increases VEGF-A expression resulting in angiogenesis and further tumor growth. Furthermore, VEGF-A is secreted from tumors, which has made it a primary objective for development of the VEGF-A antagonist bevacizumab (Avastin, Roche). Bevacizumab is used as an adjuvant chemotherapeutic agent in the treatment of metastatic colon cancer, advanced non-squamous, non-small cell lung cancer, metastatic kidney cancer, and glioblastoma. VEGF-A has also been shown to play an integral part in exudative age-related macular degeneration, where choroidal neo-vascularization results in blood and protein leakage below the macular and subsequent vision loss. Exudative age-related macula degeneration is also treated with anti-VEGF-A.

VEGF-A receptors
VEGF and its receptors are important for fetal development and vasculogenesis. Three transmembrane VEGF receptor tyrosine kinases have been identified: VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-3 does not bind VEGF-A. VEGFR-1 (Flt-1) and VEGFR-2

(KDR/Flk-1) bind all splice forms of VEGF-A and are essential for fetus survival. Both receptors are bound to endothelial cells lining normal tissue and tumor vasculature, making these cells the main target of VEGF-A (18). Especially VEGFR-2 is interesting because the binding of VEGF-A to the receptor is enhanced by the co-receptor neuropilin-1 (18,19).

VEGF-A co-receptor
Neuropilin-1 is a transmembrane glycoprotein up to 923 amino acids long (20). It consists of a large transmembrane region and an extracellular region made up of five extracellular domains. A soluble form of neuropilin-1 consisting of four out of the five extracellular domains, including the binding domains for VEGF-A and semaphorin-3A. Transmembrane neuropilin-1 acts as both a co-receptor to VEGF-A and as a receptor for semaphorin-3A. The role of soluble neuropilin-1 is unclear and somewhat complex: Monomers sequester VEGF-A and inhibit its binding to VEGFR-2, while dimers deliver VEGF-A to VEGFR-2 on the endothelial cell surface (20).

In the embryonic state, neuropilin-1 is found on neurons where it guides the growth of the nervous system through se-maphorin-3A binding. Neuropilin-1 expression on neural cells diminishes as the nervous system is developed in the embryo, but can still be found in other tissues, such as the heart and endothe-lial cells, where it promotes angiogenesis through its function as a co-receptor to VEGF-A (18,20). As with VEGF-A, neuropilin-1 expression is up-regulated by reduced blood flow and ischemia (18).

The binding of semaphorin-3A to neuropilin-1 acts as a competitive inhibitor of VEGF-A, and recent studies have shown that it also inhibits angiogenesis by inhibiting the formation of tubes from endothelial cells, as well as inducing apoptosis in the same cells at higher concentrations (21,22). Thus, the balance between VEGF-A and semaphorin-3A dictates the level of angio-genesis in meningiomas (23).

MENINGIOMA BLOOD SUPPLY AND PTBE
For a long time it was believed that PTBE around meningiomas could be explained by a physical mechanism. The consensus was that the growing meningioma would eventually compress its own venous drainage resulting in an accumulation of blood and ede-ma, which would eventually spill over into the brain. Although this has since been proven wrong, meningiomas with a well deve-loped internal venous drainage system are less likely to produce PTBE than meningiomas lacking drainage (24). Also, meningiomas with pial vascularization tend to have more PTBE than meningio-mas with external vascularization (25,26). The hypothesis is that increased intratumoral venous pressure leads to tumor con-gestion, an increase in vasogenic substances (e.g. VEGF-A and neuropilin-1), increased permeability of cerebral-pial capillaries, and expansion of the PTBE through vasogenic edema.

3. OBJECTIVES OF THE THESIS
HYPOTHESIS OF PAPER I
Based on the data on meningiomas and VEGF-A, we designed a study in order to investigate whether any correlation existed among the PTBE, edema index, VEGF-A protein, VEGF-A mRNA, capillary length, and tumor water content of primary, solitary, supratentorial meningiomas with PTBE. We investigated possible co-factors to PTBE such as meningioma subtypes, WHO grade, tumor location, and patient gender. We believe that the effect of VEGF-A on meningioma angiogenesis and the permeability of the

capillaries has a key role in the pathway that produces PTBE. We hypothesized that VEGF-A protein and mRNA were correlated to increased capillary length, tumor water content and PTBE in meningiomas, and that capillary length would also be correlated to increased PTBE.

HYPOTHESIS OF PAPER II
We ran several test assays to ensure that the chemiluminescence method for mRNA quantification was acceptable, before using the method in paper I. Since studies comparing the chemilumi-nescence method with the usually preferred method for mRNA quantification, RT-qPCR, were sparse, we chose to investigate this matter further. We hypothesized that a comparison between the Panomics Lumistar's chemiluminescence and the RT-qPCR assays' abilities to quantify GADPH and VEGF-A in intracranial meningio-mas would produce similar results in regards to the precision and reproducibility.

HYPOTHESIS OF PAPER III
Paper I showed a correlation between VEGF-A and brain edema as well as capillary length and PTBE. Too few angiomatous and secretory meningiomas were available in order to conclude whether they did in fact produce more PTBE than non-angiomatous meningiomas. We hypothesized that with a suffici-ent sample size, it would be possible to confirm that angiomatous and secretory meningiomas had larger PTBE than non-angiomatous meningiomas and that not only VEGF-A, but also its receptors VEGFR-2 and neuropilin-1 were correlated to the PTBE formation. We also hypothesized that the gender specific findings in paper I were due to a correlation between the expression of female sex-hormone receptors and the PTBE.

4. METHODOLOGICAL CONSIDERATIONS
Tissue samples were collected from patients operated at the Neurosurgical department of the Copenhagen University Hospital. Tissue samples collected throughout the period 1999–2012 were analyzed in this thesis. As we started the study in 2007, the largest body of samples was collected prospectively in 2007–2008, but later we had to systematically search for angiomatous and secretory meningiomas, in which case we searched as far back as 1999. In order to avoid confounding factors, the included meningioma patients had primary and solitary tumors. The pati-ents had not received radiation therapy prior to diagnosis and surgery.

ASSESSING THE TUMOR SIZE AND BRAIN EDEMA
MRI of the brain produces images with high contrast levels bet-ween normal and edematous brain tissue. MRI also produces axial, sagittal, and coronal images of the brain, and sagittal or coronal images are essential in addition to axial images for accu-rate tumor and PTBE measurements. Today, most patients with brain tumors undergo an MRI-scan prior to surgery, but this was not the case in 2007. At the time, radiology departments had to prioritize patients with malignant brain tumors. It was therefore not possible to acquire MRI of all patients with meningioma, and a number of patients only had a CT-scan prior to surgery. CT-scans with infused contrast produce images with excellent contrast levels between the brain tumor and the surrounding PTBE. The visualization of the size and localization of the tumor was suffici-ent for surgery, but the contrast level between normal and ede-matous brain tissues was not precise enough for measuring the

size of the PTBE. In addition, the contrast enhancement is not specific to PTBE and can be due to the tumor itself, increased peritumoral vasculature or inflammation. The standard CT protocol neither included sagittal nor coronary images. Ultimately, we chose MRI for assessing the tumors and PTBE, feeling confident this would produce the most accurate, and reproducible tumor and PTBE measurements.
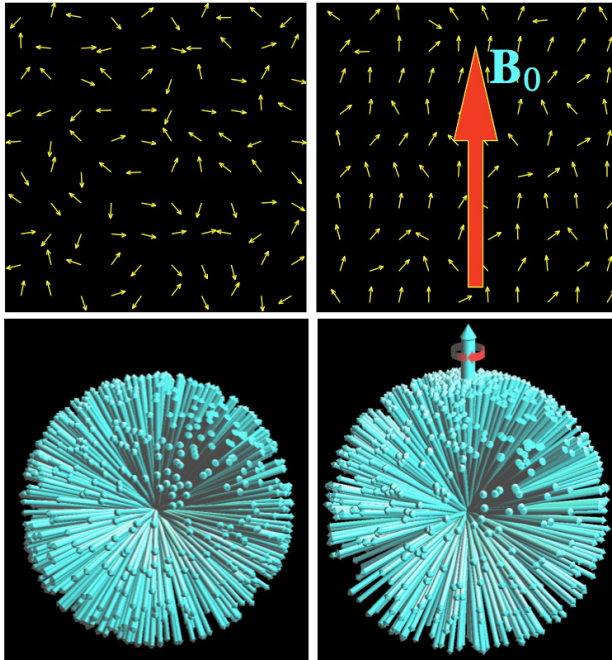

Figure 1
The magnetization of nuclei in a subject before (left) and during (right) magnetic resonance imaging (MRI), shown in 2D (upper figures) and 3D (lower figures). Before the magnetic field is applied (left), the nuclei are spinning with a uniform distribution, i.e. the net magnetization is near zero. When a magnetic field (B0) is added, the distribution becomes skewed resulting in a net magnetization represented by the thick arrow in the lower right figure. Figure presented with permission from Lars G. Hanson (66)

Although an MRI-scanner produces sliced images just like a CT-scanner, it works very differently. The most notable difference being that no harm is done to the patient since he or she is not exposed to radiation. Instead, multiple powerful magnets apply a strong magnetic field around the patient. The magnetic field aligns the spinning protons of the water molecules in the patient (Fig. 1). The MRI-scanner then shortly emits a different magnetic signal, which flips the spin of the protons to a new state. When the signal is turned off, the protons flip back to the original, relaxed, state while emitting a radio frequency (27). The frequency is measured by signal coils and analyzed by the MRI-computer based on the speed with which protons in different tissues return to their relaxed state. T1- and T2-images are based on the time it takes for the proton to return to its relaxed state in the z- and xy-planes, respectively. T1-images differentiate well between water and fat, therefore they were used for assessing the tumor size. Intra-venous (i.v.) contrast can be administered for better visualization of tumor borders and PTBE. As with contrast-based CT-images, contrast-based T1-images are not specific for PTBE and cannot differentiate between factors of increased peritumoral fluid concentration. T2-based MRI-scans have a high affinity for contrasting water and brain tissue and were used for assessing the PTBE. In order to better differentiate the PTBE, both T1- and T2-images used FLAIR (fluid attenuated inversion recovery). An

inversion-recovery pulse signal was used to further null the water signals using an inversion time (TI) based on the T1- and T2-values of the MRI-scans, effectively nulling out the signals from the cerebrospinal fluid. Diffusion weighted images intensified local pixels in order to better present subtle changes in water diffusion rate. Because of the tract-like structure of the central nervous system, and the extracellular waters diffusion along these tracts, trace apparent diffusion coefficient (tADC) images were prepared. With tADC-images it was possible to assess the structure of the tumor, e.g. cystic vs. solid, so that cystic parts of tumors were not calculated as PTBE. Thus, tumor size with and without PTBE was assessed on axial, coronal and sagittal MRI before surgery. More specifically, MRI were obtained with a 3.0 T scanner with a 12-channel head-coil (Magnetom Trio MR B15 Siemens). The MRI-protocol included five sequences; a sagittal T1-weighted fluid-attenuated (FLAIR) sequence with nineteen 5.0 mm slices (TR/TE/TI; 2000 ms/23 ms/800 ms), a coronal T2-weighted fluid-attenuated (FLAIR) sequence with 5 mm slices (TR/TE/TI; 9000ms/77ms/2500 ms), an axial T2-weighted blade FSE (fast-spin-echo) sequence with twenty one 5.0 mm slices (TR/TE; 4000 ms/89 ms), an axial diffusion weighted echo planar sequence with twenty two 5.0 mm slices encoded in x, y and z directions (TE/ b0, b1, b2; 104 ms/ 0, 500 & 1000 s/mm²), and a sagittal 3D magnetization preparation rapid imaging (MPR) sequence with 192 partitions of 1.0 mm (TR/TE/TI; 2250 ms/3 ms/900 ms) after i.v. contrast media (Multihance 0.1 mmol/kg). A trace apparent diffusion coefficient (tADC) map was calculated based on the three b values encoded in three directions (x, y & z). Axial reformated 3.0 mm images were calculated from the 3D MPR data set.

We used the formula $V = \frac{4}{3}\pi abc$ to calculate volumes of tumor (V_{Tumor}) and tumor with PTBE ($V_{Tumor\ +PTBE}$), respectively. The PTBE (V_{PTBE}) was then calculated as $V_{PTBE} = V_{Tumor+PTBE} - V_{Tumor}$. In order to remove the confounding effect of tumor size, we calculated an edema index as $\text{edema index} = \frac{V_{PTBE+Tumor}}{V_{Tumor}}$. Tumors without PTBE would thus have an edema index of 1.

MRI shows the extent of PTBE in the brain. Although it is not a direct measure for the water concentration in the brain, it is interpreted as such, because of the limited access to peritumoral brain tissue. As tumor tissue was readily available, it was possible to measure the tumor water content. A low-tech technique for measuring the water content of the meningiomas was used. By heating the tissue samples to 60 ºC for 48 hours, we were able to measure the total and dry weights of the tissue samples. The water-weight ratios could then be calculated. For this method at least 40 mg of frozen tissue was needed. Smaller samples and tumors kept in tissue-tech could not be measured. The smaller tumors would shrivel up and the tissue-tech added an unknown weight and water ratio to the tissue. As the tissue tubes would also lose water and weight during the incubation, we measured five test tubes before and after incubation in order to calibrate our results.

CONTROL BRAIN TISSUE
Because of ethical and practical considerations, it was not possible to procure neither normal brain tissue samples from healthy test subjects nor samples of brain tissue adjacent to the meningiomas. It was not feasible to use meningeal tissue samples either, as the meninges are mostly made up of connective tissue with very few cells. Instead, tissue samples were collected from temporal-lobe resected epilepsy-patients. These patients under-

went temporal lobe resection as a last-resort therapy when medical treatment was shown to be inadequate. Surgery was based on electroencephalography, neurological examination, and MRI to ensure as few complications as possible. In cases where pathological features were not present in the resected tissue, samples were chosen as controls for comparison with the meningiomas. Although not perfect, we believe that this approach was the most optimal, and has also been used by others (28).

HISTOLOGY
Immunohistochemistry is a process for visualization of proteins by binding of animal antibodies specifically to target human antigens in biological material. Traditionally an antibody is conjugated to an enzyme that catalyzes a color-producing reaction after the tissue section has been immunostained with the antibody (Fig. 2).
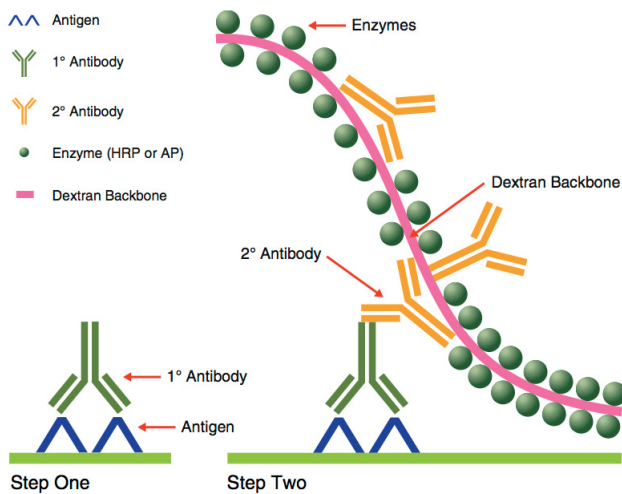

Figure 2
By utilizing polymer-based immunohistochemistry, target antigens can be visualized on tissue sections in a few steps. First, a specific, primary antibody binds to the target antigen. During the next step, the secondary antibody is added along with a polysaccharide (dextran) backbone and horse-radish-peroxidase (HRP), which conjugate to the secondary antibody. The secondary antibody is specific to the primary antibody, thus indirectly labeling the target antigen. A substrate which reacts with HRP is added for staining. Finally, a counterstain is provided to provide contrast with the primary stain. (67)

Immediately after surgery, one tissue bloc was frozen in liquid nitrogen and stored at minus 80 ºC. The adjacent bloc was formalin-fixed and embedded in paraffin for immunohistochemistry. Tumor sections, 4 µm thick, were used for diagnosis (HE) and immunohistochemistry. The sections for immunohistochemistry were pre-treated in a microwave oven with a tris-EGTA-buffer, and immunostained for CD34 (monoclonal mouse anti-human CD34 class II, DAKO, Glostrup, Denmark, No. M 7165, 1:400), MIB-1 (monoclonal mouse anti-human Ki-67 antigen, DAKO, Glostrup, Denmark, No. M 7240, 1:100), estrogen (monoclonal mouse anti-human Estrogen receptor α, DAKO, Glostrup, Denmark, No. M 7047, 1:50), and progesterone receptors (monoclonal mouse anti-human Progesterone receptor, DAKO, Glostrup, Denmark, No. M 3569, 1:50) on a DAKO Cytomation autostainer. Meningiomas were classified independently by two neuropathologists (HL and HB) according to the WHO criteria (12).

During the histological assessment of the tissue samples, great care was taken to follow identical procedures for all samples. Cells were counted on sections immunostained for MIB-1. The MIB-1 index was estimated on sections as the percentage of

MIB-1 positive tumor cells counted in 10 HPF (high power field = 0.0469 mm^2) in five parts of the tumors. Cross sections of intra-tumoral capillaries were counted on sections immunostained for endothelial cells with CD34-antigen. The capillary length in mm per mm^3 of tissue was calculated from the microvascular density using Gundersen's (29) formula:

$$L_{capillary} = 2 \cdot Q_A \cdot V \Leftrightarrow \frac{L_{Capillary}}{V} = 2 \cdot Q_A = \text{capillary length in mm per mm}^3$$

. The microvascular density was estimated as the average counts of cross-sectioned capillaries (Q_A) per mm^2 on 10 HPF measured in five "hot spots" with high vascular density.

One hundred tumor cells were counted in the upper left corner of ten randomly selected grid fields on sections immunostained for estrogen and progesterone receptors. The receptor indices were estimated as the percentage of positively stained tumor cells in each section.

GENE EXPRESSION ANALYSES
Depending on the function of the cell and its current needs, specific parts of its DNA is transcribed to mRNA which acts as a template for translation during protein synthesis. Through the process of translation, amino acids are combined in sequences dictated by the mRNA and ultimately the DNA of the cell. The mRNA is easily degraded, mainly by RNAse, both in vivo and in vitro. Special care was taken with the tissue samples in order to ensure that mRNA was not lost. In order to ensure the mRNA, all samples were snap-frozen in liquid nitrogen after surgery and stored at minus 80 ºC. Biological processes were halted at this low temperature. At times when tissue samples were needed, special care was taken to keep the samples on dry ice during the process and to return them quickly to the freezer.
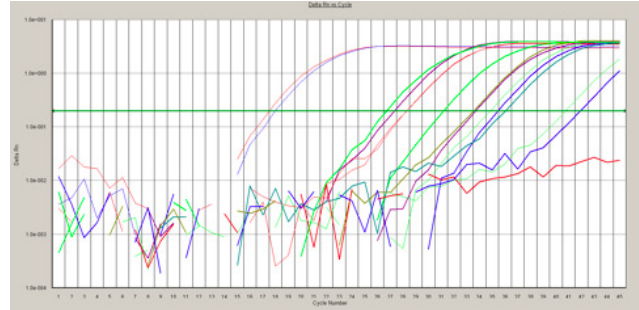

Figure 3
An example of a RT-qPCR assay. Each curve represents a PCR, which rises depending on the number of copies of the sample target. The cycle number decreases with increasing amount of target. Since PCR is an exponential amplification where each cycle ideally doubles the target amount, data can be converted to linear data by the formula $2^{-Ct} \cdot 10^9$ where Ct is the cycle-value at the intersection between the threshold line and the amplification curve.

The preferred method for mRNA measurement is usually quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), where RNA is isolated, measured, reverse transcribed into complementary DNA (cDNA), purified, and amplified via "real time"-PCR: The cDNA is combined with a DNA dependent polymerase and fluorescence-labeled oligonucleotide primers, enabling amplification of the cDNA. The fluorescence level is monitored while the DNA amount ideally doubles with each amplification cycle (PCR cycle). When the amplification rate becomes exponential, and the amplification curves become parallel, the Ct-value is noted, based on the intersection between
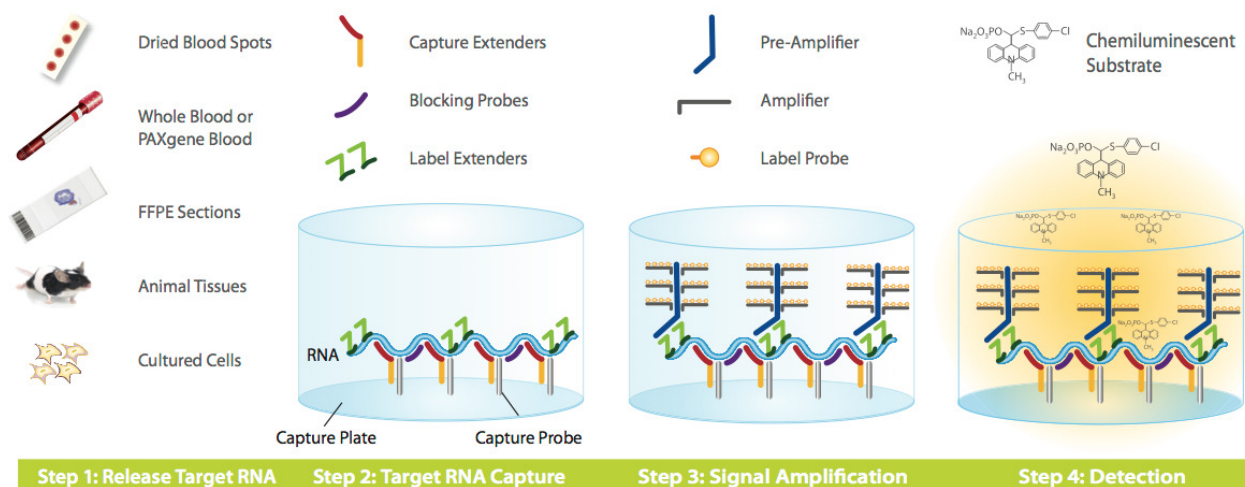
Figure 4
The Lumistar assay is based on quantification of the mRNA targets built upon the branched DNA (bDNA) technology, which relies on cooperative hybridization between a target mRNA and a target-specific probe set which uses bDNA to amplify the mRNA signal. Figure presented with permission from Affymetrics-Panomics Solutions, Santa Clara, CA, U.S.A.

amplification curves and the threshold line. The Ct-values can then be used to determine the relative starting amount of RNA (Fig. 3). The PCR assay is known as robust and reliable (30), although practical alternatives could be expected to challenge PCR, if some of the laborious procedures could be omitted (31).

When possible, a simpler method was used for most of the mRNA analyses. The principle of the method was to capture the mRNA in cell lysates with DNA-probes immobilized on a solid support, attached via generic DNA-probes. Detection could be done either on the surface of beads or, as in our study, micro-titer wells (Fig. 4). Tissue material only had to be homogenized and treated with proteinase K before being transferred to the wells. The detection of mRNA was implemented by the addition of protection probes, to avoid unspecific hybridization, and detection probes, termed branched DNA (bDNA), conjugated to horseradish peroxidase (HRP), and finally addition of a chemiluminescent substrate. The usage of bDNA implements a sort of amplification in the eventual chemiluminescence reaction because each detection probe is attached to numerous HRP-molecules. Detection is finally performed with a luminometer and the level of chemiluminescence semiquantitatively represents the concentration of mRNA. A series of assays based on chemiluminescence were run before study I, but because of the sparsity in the literature comparing the method with RT-qPCR, we ran a larger study to ensure the precision and reproducibility of the assay. RT-qPCR was only used for VEGFR-2 gene expression analysis as chemiluminescence probes for VEGFR-2 were not available.

When comparing gene expression in various samples, the challenge is to normalize mRNA quantities so that a specific and reliable measure for gene expression can be achieved. In paper I, we used three housekeeping genes (β-actin, 28SrRNA, and GAPDH) in order to ensure the best possible normalization. There is a consensus that housekeeping genes have a near constant concentration in all cells, and are therefore used for normalization of target mRNA. In our later papers, it was decided to prioritize efficiency by limiting the number of housekeeping genes to GAPDH. This housekeeping gene was chosen for two reasons. Most importantly GAPDH followed the normal distribution more

closely than β-actin, which meant that the GAPDH values were more stable. Secondly, 28SrRNA needed dilution, which induced a time consuming step which was prone to human error. Furthermore, GAPDH has been used for normalization for some time and a larger study has shown that the GAPDH mRNA levels are very constant in similar tissues, e.g. brain tissue (32). The drawback of using GAPDH was that the luminescence level was higher than VEGF compared to both β-actin and 28SrRNA. In some cases this would lead to overexposed sample results which had to be re-analyzed at a lower concentration.

PROTEIN ANALYSES
Since proteins are more robust than mRNA, protein analysis was less delicate. We used enzyme-linked immunosorbent assays (ELISA) for measuring VEGFR-2, neuropilin-1, and total protein. In the case of VEGF-A protein we used chemiluminescent probes for protein assessment instead of enzyme-linked probes. All assays involved immobilization of the target proteins in detection wells on an ELISA-plate by using target-specific antibodies, after which substrate was added in order to produce either an enzyme-linked color change or chemiluminescence which was then detected by either a spectrophotometer or a luminometer. A known dilution series was added to each plate in order to calculate the amount of protein in each sample. In order to compare the sample results, total protein levels were used for normalization. Total protein levels are regularly used for target protein normalization, and have been shown to produce consistent results (33). A single tissue lysate from each tissue sample was used for quantification of all of the proteins for each sample.

CRITICAL APPRAISAL OF SAMPLING AND METHODS
It is important to consider several types of bias in our studies. Selection bias was possible because of the choice to only include meningioma patients with MRI: One might risk leaving out patients with a very dramatic tumor presentation, in cases where there was no time for MRI and only CT-scanning was performed prior to surgery. Also, a meningioma might have a high potential for PTBE formation, but because of its location it would be disco-

vered and removed early—before PTBE formation. The possibility for this selection bias was removed by comparing the non-angiomatous and angiomatous meningioma groups in paper III. Fifteen of the twenty-two angiomatous and secretory meningiomas had available CT-scans or MRI, and thirteen of the fifteen had PTBE. We feel that the decision to also include angiomatous and secretory meningiomas without MRI in paper III was well founded. In order to avoid investigator bias, the MRI-evaluator was blinded to the meningioma diagnosis and patient history.

Since the meningiomas were removed by several different surgeons, because the meningiomas were not always presented to the neuropathological department in one piece, and because they were randomly dissected by the technician, there was no room for stereological bias during dissection. The fields for microscopic assessment of the estrogen- and progesterone-receptor indices were chosen systematically at a macroscopical level, and all microscopy was performed without knowledge of the histological diagnosis, i.e. blinded. Therefore investigator bias was minimized, also during capillary length assessment.

Meningiomas are very homogenous tumors. Despite of this, special care was taken during tissue sampling for protein and mRNA analyses, not to sample tissue areas with hemorrhage or meninges in order to sample as homogenous tissue samples as possible. As tissue density differed between the tissue samples, exact weighting of the samples did not have any purpose as two samples with identical weights could contain a vastly different number of cells. Instead the protein and mRNA quantification results were normalized to total protein and housekeeping genes as described earlier. This removed the potential quantification error. It should be noted that in the case of several up-regulated proteins in addition to the target protein, existing differences will become less clear after normalization as the relative target-protein/total-protein does not increase significantly.

5. SUMMARY OF OWN STUDIES
PAPER I
During a two year period, starting from january 2007, tissue samples from 101 meningioma patients undergoing treatment at the Department of Neurosurgy of the Copenhagen University Hospital were collected for further investigation. The primary objective of the study was to investigate VEGF-A and PTBE in intracranial meningiomas, but also to test whether frontal and angiomatous meningiomas had a higher tendency for developing PTBE. For this purpose, patient records and MRI scans were examined. Furthermore, tumor sections were frozen for later VEGF-A protein and mRNA study while adjacent sections were prepared for histological diagnosis and capillary length measurement. Because MRI only showed the extension of the PTBE, water content analysis was also performed on the tissue samples in order to assess the intratumoral water level. Since cortical tissue samples from healthy individuals were not available, tissue samples without pathological findings from temporal-lobe resected epilepsy-patients were used as control brain tissue samples.

After thorough testing it was decided to use a new chemiluminescence method as it was less laborious in comparison to the traditional RT-qPCR method for quantification of the VEGF-A mRNA.

Anecdotal evidence shows that frontal meningiomas produce more PTBE. Our study showed this general assumption to be false. Furthermore, meningiomas were shown to have longer capillaries, higher water content, VEGF-A protein and mRNA compared to the control brain tissue samples, suggesting that

angiogenesis is increased in meningiomas, that the meningioma capillaries are possibly more permeable than in the control brain samples, and that the meningiomas secrete both VEGF-A protein and mRNA. Because of small sample size, it was not possible to make any conclusions regarding angiomatous and secretory meningiomas.

Infratentorial meningiomas rarely develop PTBE, probably because of their localization in the posterior fossa, where even small tumors result in affection of the cranial nerves or the brain stem. Thus, they quickly present themselves and are removed before significant PTBE can develop. These meningiomas were therefore not included in the second part of the paper were VEGF-A and PTBE were compared. Also, because factors like genetics, prior surgery, and treatment with whole-brain radiotherapy could have confounding effects on the level of VEGF-A and PTBE, 43 primary, solitary, supratentorial meningiomas with brain edema of the initial 101 meningioma patients were included in the testing of the primary hypothesis of the paper: Is PTBE increased in meningiomas with high levels of VEGF-A. The study showed a significant correlation between VEGF-A and the edema index, and capillary length was correlated to PTBE. Furthermore, female patients had higher levels of meningioma water content compared to male patients, while the latter had higher levels of VEGF-A protein.

PAPER II
We wished to ascertain whether the chemiluminescence assay was as reliable as RT-qPCR. Ten angiomatous meningiomas and five control brain tissue samples were analyzed using both RT-qPCR and chemiluminescence (Lumistar). Angiomatous meningioma and control brain tissue samples were chosen in order to test VEGF-A and GAPDH mRNA levels in tissues with high and low expression levels.

We found that the Lumistar-signal decayed over time, but that the results were consistent if the reading time was kept constant. Also, the dynamic range of Lumistar was narrower compared to RT-qPCR (10^{-9} or more). Dilution series with a known arbitrary concentration of GAPDH were prepared for both RT-qPCR and Lumistar. The results showed significant quantitative relationship between GAPDH output and arbitrary concentration values for both methods. In addition, VEGF-A was normalized to GAPDH using both RT-qPCR and Lumistar. The results showed significant correlation.

We found that Lumistar was competitive to RT-qPCR, and reflected a similar pattern of mRNA measurement with a similar precision. The preferred method for mRNA quantification should therefore be chosen based on the variability of the samples, budget, and time: RT-qPCR was more robust in case of severe sample inter-variability, and the assay was less expensive. Lumistar on the other hand was less time consuming and more adaptable as formalin-fixed and paraffin-embedded (FFPE) tissue samples could easily be analyzed.

PAPER III
We continued the study of the VEGF-A induced PTBE pathway by examining the expression of VEGFR-2 and neuropilin-1 in the same supratentorial meningiomas. The same control brain tissue samples from paper I were used for comparison. As angiomatous and secretory meningiomas are though to be prone to induction of PTBE, a systematic search was performed for supratentorial, angiomatous and secretory meningioma tissue samples. Nineteen additional angiomatous and secretory meningioma tissue samples

were added to the dataset for a total of 22 angiomatous and secretory meningiomas, for further study of the PTBE and the gene expression of VEGF-A, VEGFR-2, and neuropilin-1. Finally, following the gender specific differences in meningioma water content, we investigated the samples for a possible correlation between PTBE, the VEGF-A pathway, and the estrogen and progesterone receptor expression.

VEGF-A mRNA, VEGF-A protein, and neuropilin-1 mRNA were higher in angiomatous and non-angiomatous meningiomas compared to controls. VEGFR-2 protein was higher, and neuropilin-1 protein lower in angiomatous meningiomas compared to controls. The mean capillary length was 3614 mm/mm^3 in angiomatous, 605 mm/mm^3 in non-angiomatous meningiomas, and 229 mm/mm^3 in the controls. Angiomatous meningiomas were 3385 mm/mm^3 ($p < 0.0001$), and non-angiomatous meningiomas 376 mm/mm^3 ($p = 0.014$) longer than controls.

Forty patients with non-angiomatous and ten patients with angiomatous meningioma had pre-operative MRI exams. The two groups had equally sized tumors. The mean PTBE area around the angiomatous meningiomas was 695 cm^3, i.e. 477 cm^3 larger than the non-angiomatous meningiomas ($p = 0.0045$), and the mean edema index was twice the size compared to the non-angiomatous meningiomas.

Forty non-angiomatous and twenty-two angiomatous meningiomas were analyzed for VEGF-A, VEGFR-2, and neuropilin-1 mRNA and protein levels; capillary length; and MIB-1 indices. Mean VEGF-A mRNA, VEGFR-2 protein, and neuropilin-1 mRNA were significantly higher and neuropilin-1 protein was lower in the angiomatous meningiomas. Mean capillary length was 3614 mm/mm^3 in angiomatous meningiomas, i.e. 3009 mm/mm^3 longer than in the non-angiomatous meningiomas ($p < 0.0001$). The other parameters did not differ between the groups.

No meningiomas showed any particular immunostaining (< 1%) for estrogen receptors. In the non-angiomatous group, meningothelial (median 87%, range 21–96%) and fibromatous meningiomas (median 3%, range 1–11%) had significantly high and low progesterone expression levels ($p < 0.001$). The angiomatous meningiomas presented themselves in subgroups with either low (median 4%, range 0–22%) or high (median 88%, range 79-96%) progesterone expression. The difference between these subgroups was significant ($p = 0.0003$). Nevertheless, sex-hormone receptors were not correlated to the VEGF-A pathway or PTBE formation in any way.

6. DISCUSSION
CONTROL TISSUE
Due to the limitations in procuring control tissue samples, this group differed (median age 37 years, range 9–50; 30/70 female/male ratio) from the meningioma population in median age and gender distribution. The control patients were younger and were predominantly male.

MENINGIOMAS
The meningioma sample in paper I closely resembled what was expected from a random sample of a population with meningiomas in regards to median age (61 years) and gender distribution (65/35 female/male ratio) (2). Of the 79 patients with MRI, 41 had frontal meningiomas, and nine were infratentorial (Table 1). The histological grade distribution of the meningiomas was as expected (2), with a distribution as follows: Grade I N = 86, grade

II N = 12, and grade III N = 3 (Fig. 5). In the subgroup of 43 supratentorial meningioma patients with PTBE, the median age was unchanged (60 years), and the gender distribution was still predominantly female (60/40 female/male ratio). There was no grade III meningiomas in this group, but the distribution of grade I (N = 39) and grade II (N = 4) meningiomas stayed practically unchanged with 90% in the subgroup vs. 85% grade I meningiomas in the main group.
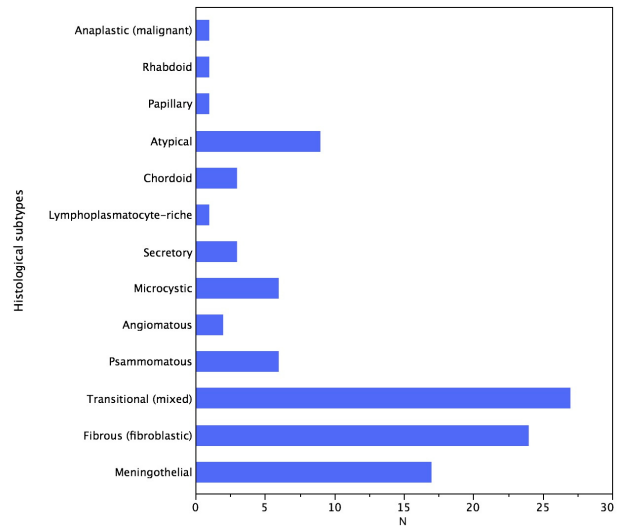

Figure 5
Two neuropathologists (HL and HB) independently classified all meningiomas (paper I) by the WHO criteria. Chordoid and atypical meningiomas are WHO grade II tumors while papillary, rhabdoid, and anaplastic meningiomas are WHO grade III tumors. The rest of the subtypes are WHO grade I tumors.

Steroid therapy
Many of the patients with meningiomas with PTBE received steroid therapy within three days before surgery. MRI was usually taken prior to this time. As steroid therapy is believed to reduce the PTBE by temporarily reducing the permeability of the tight junctions in the blood-brain-barrier (34-36), and possibly also VEGF-A in meningioma cells (37), it was interesting that we did not find any difference in VEGF-A protein or mRNA or tumor water content in paper I. Tumor size and PTBE was significantly higher in patients who received steroid therapy, but the edema index was not significantly different. In the clinical setting, patients with symptoms of increased intracranial pressure are usually administered steroid therapy. It is very plausible that patients with increased tumor size and PTBE had more profound symptoms compared to other patients, and they were therefore more likely to receive steroid therapy. As there was no difference in edema index or any other factors between patients who did og did not receive steroid therapy, we do not believe that this was a confounding factor.

Location
Although others have noted that frontal meningiomas have a tendency towards PTBE formation (38), this could not be found in paper I. Instead we found that supratentorial meningiomas had larger PTBE (102 vs. 72 cm^3, $p = 0.019$) and edema index (2.29 vs.

Table 1

Meningioma localization (N) – data from paper I

Lobe	Infratentorial	Convexity	Basal	Falx	Total
Cerebellar	9	-	-	-	**9**
Frontal	-	12	23	6	**41**
Temporal	-	3	7	-	**10**
Parietal	-	5	-	8	**13**
Occipital	-	3	-	-	**3**
Suprasellar	-	-	1	-	**1**
Orbital	-	-	2	-	**2**
Total	**9**	**23**	**33**	**14**	**79**

1.41, p = 0.018) compared to infratentorial meningiomas. We believe that the constricted space of the fossa posterior contributes to this difference between supra- and infratentorial meningiomas. As supra- and infratentorial meningiomas were of the same size (193 and 191 cm^3 respectively), we believe that the formation of PTBE around the infratentorial meningiomas resulted in earlier symptoms, e.g. cranial nerve palsies. Because of earlier diagnosis and surgery, this group would naturally present itself with a smaller PTBE and edema index in our study. As mean capillary length and tumor water content were statistically equal (p > 0.05) in supra- (water content 84%, capillary length 637 mm/mm^3) and infratentorial meningiomas (water content 82%, capillary length 482 m/mm^3) we feel that our assumption is further substantiated. To our knowledge, only Otsuka et al. (39) have quantified VEGF-A and the edema index in supra- and infratentorial meningiomas, but we were unable to compare our results, as they did not comment on that part of their dataset.

Meningiomas with and without PTBE

When comparing the supratentorial meningiomas with and without PTBE, we only found that the meningiomas with PTBE were substantially larger (median tumor size 215 vs. 17 cm^3; p < 0.001). Although Sakuma et al. found increased VEGF-A protein in supratentorial meningiomas with PTBE compared to those without PTBE (40), we could not find this difference in paper I. The sample size of supratentorial meningiomas without PTBE was smaller in our study (five out of 48 supratentorial meningiomas), compared to Sakuma et al.'s study, which included 21 supratentorial meningiomas without PTBE out of a total of 40 meningiomas. After having published paper I, we further scrutinized Sakuma et al.'s data by statistical calculation. We found that the statistical method used by Sakuma et al. (one way analysis of variance) for comparison between supratentorial meningiomas with and without PTBE was not sound. One-way ANOVA assumes that each data group is approximately normally distributed and that the variability within the groups is roughly constant. This is not the case with the edema index, capillary length, or VEGF-A protein data in either our own or Sakuma et al.'s study. Therefore, we have repeatedly used a non-parametric method (e.g. Kruskal-Wallis H-test) for comparison between categorical groups, or log2-transformed the data into a normal distribution. Using non-parametric statistical tests to correctly analyze Sakuma et al.'s data, we found that the higher VEGF-A protein level in the supratentorial meningiomas with PTBE was without statistical significance.

Capillary length and VEGF-A

Only Sakuma et al. (40) and ourselves have properly examined capillary length and VEGF-A in meningiomas. In paper III, VEGF-A mRNA and capillary length were significantly increased in angiomatous compared to non-angiomatous meningiomas and controls (Table 2). VEGF-A protein was significantly increased in angiomatous and non-angiomatous meningiomas compared to controls, and capillary length and VEGF-A mRNA were higher in non-angiomatous meningiomas compared to controls (Table 2). These results lead us to believe that VEGF-A protein and mRNA are increased along with capillary length in meningiomas, especially in angiomatous meningiomas. As VEGF-A is important in angiogenesis, the increased capillary length is probably due to the higher VEGF-A protein and mRNA levels.

VEGF-A and PTBE

VEGF-A mRNA was positively correlated to the edema index (p = 0.03) for both genders, and VEGF-A protein was correlated to the edema index in female patients (p = 0.0014) in paper III. In addition to our own and Sakuma et al.'s studies, a few other groups have investigated the relationship between VEGF-A and PTBE in meningiomas. In 1997, Goldman et al. retrospectively investigated 37 patients with meningioma for VEGF-A (41). Only 26 patients had PTBE on MRI. They were graded into groups based on subjective levels of PTBE, as the "actual pixel data from scan tapes could not be accessed". Goldman et al. found a significant positive correlation between VEGF-A protein and MRI edema rating, i.e. PTBE (41). Paek et al. performed a study in 2002 of 20 meningioma patients of which 10 had PTBE verified on MRI (42). VEGF-A protein level was quantified by western blotting and semi-quantified by immunohistochemistry. Although VEGF-A protein was increased in meningiomas with high edema indices, the results were not significant, probably due to the small sample number of meningiomas with PTBE. In 2004 Otsuka et al. retrospectively studied 118 cases of meningioma patients (39). The PTBE was visualized on MRI and tissue sections were immunostained for VEGF-A and graded into four groups. They found that high expression of VEGF-A protein was correlated to a high edema index. Ding et al. studied 40 patients with supratentorial meningiomas. The PTBE was measured on MRI, VEGF-A protein and mRNA were quantified using western blotting and RT-qPCR (28). Ding et al. sampled 5 mm^3 peritumoral tissue from each patient. They also used temporal-lobe resected tissue-samples from epilepsy patients as control tissue (N = 5). Ding et al. found a positive correlation between VEGF-A mRNA and protein and the edema index. They also found that VEGF-A mRNA and protein were 10 times higher in meningiomas than in controls. In the peritumoral tissue samples, the VEGF-A mRNA

Table 2

Mean and range data from paper III (*p < 0.05)

	Control	Non-angiomatous	Angiomatous
Capillary length (mm/mm^3)*	229 (117–443)	605 (27–3644)	3614 (433–42671)
MIB-1 (%)	-	0.94 (0–5.95)	0.42 (0–3)
VEGF-A protein*	28.71 (13.10–90.53)	111.49 (17.58–2470.17)	79.34 (4.09–1051.48)
VEGF-A mRNA*	16.56 (9.92–27.23)	68.26 (12.29–421.20)	131.42 (5.42–893.00)
VEGFR-2 protein (10^{-5})*	5.15 (2.90–8.58)	6.31 (1.95–25.46)	71.9 (3.46–557.69)
VEGFR-2 mRNA	0.14 (0.009–4.06	0.04 (0.001–2.13)	0.04 (0.00002–0.97)
Neuropilin-1 protein (10^{-5})*	33.46 (14.20–56.76)	36.33 (12.85–99.20)	5.64 (0.60–43.47)
Neuropilin-1 mRNA*	8.19 (4.38–13.86)	66.35 (17.50–200.71)	89.02 (16.97–365.56)
Tumor size (cm^3)	-	226 (23–1266)	221 (65–1203)
PTBE (cm^3)*	-	218 (16–2039)	695 (179–1787)
Edema index*	-	2.25 (1.10–13.56)	4.61 (1.47–16.31)
N total	10	40	22 (10)

levels were just as low and the VEGF-A protein levels five times higher than in the epilepsy control samples, whereas the VEGF-A protein levels were lower than in the meningiomas. In our studies both VEGF-A protein and mRNA were increased in angiomatous and non-angiomatous meningiomas compared to controls, and VEGF-A mRNA was significantly more increased in angiomatous than non-angiomatous meningiomas (Table 2). Interestingly, in 1997 Goldman et al. stated that the VEGF-A immunostaining of the outer cells of the meningioma tumors were more positively correlated to the PTBE than the inner-most cells (41). In combination with our own findings of increased capillary length in non-angiomatous, and especially angiomatous meningiomas (Table 2), along with the 0.1–4.3% higher water content in the supratentorial meningiomas with PTBE compared to the controls (mean water content 84% vs. 82%, p = 0.01), we believe that this strongly supports the assumption that the meningiomas produce and secrete VEGF-A protein to the peritumoral brain. We also believe that the rise in capillary length, water content, and PTBE is for the most part a result of this increased VEGF-A level.

Capillary length, pial blood supply, and PTBE
Sakuma et al. did not find a correlation among the capillary length and the PTBE or edema index (40), but, probably because we had a larger sample size of supratentorial meningiomas with PTBE (N = 43) compared to Sakuma et al. (N = 19), we were able to find a positive correlation between the capillary length and the PTBE (p = 0.038). In addition to longer capillaries, angiomatous meningiomas (N = 10) had larger PTBE compared to non-angiomatous meningiomas (N = 40) (Table 2) in paper III. In 2010, Schmid et al. (43) published a study of 79 intracranial, grade I meningiomas that showed an increased PTBE in meningiomas with a pial blood supply as well as positive VEGF-A immunostaining (p < 0.002). The level of VEGF-A staining was not quantified, but the size of the study makes it interesting. The meningiomas without pial blood supply and VEGF-A had a significantly lower mean edema index[1] compared to the meningioma group with pial blood supply and positive VEGF-A staining (1.4 ± 1.3 vs. 2.5 ± 1.3, p < 0.008). In 2012, Iwado et al. (44) studied the relationship between pial blood supply, VEGF-A protein and PTBE in 60 patients with meningiomas. Iwado et al. had categorized the meningiomas into four groups based on VEGF-A immunohistochemistry. Pial blood supply and VEGF-A protein were independently, and positively correlated to the PTBE. Furthermore, pial blood supply was also

correlated to the tumor size and the VEGF-A protein level. As our own and Sakuma et al.'s (40) studies have shown that capillary length is increased in angiomatous meningiomas, and that the capillary length is correlated to the VEGF-A protein level, we believe that the correlation between the pial blood supply and the PTBE is confounded by the fact that increased VEGF-A increases angiogenesis as well as PTBE. This notion is further substantiated by the fact that more than half (N = 46) of the meningiomas with VEGF-A immunostaining in Schmid et al.'s study (43) did not have a pial blood supply, but still had a significantly large PTBE (43). Furthermore, Otsuka et al. did not find a significant correlation between pial blood supply and edema index in intracranial meningiomas (39). It is possible that there is a causative connection between pial blood supply and tumor size, since Schmid et al. found significantly larger tumor size in the meningiomas with pial blood supply compared to those without (43).

VEGFR-2
A brazilian study written in portuguese by Souto et al. in 2002 (45), was evaluated after translation of the paper through translate.google.com. The study included 21 meningiomas immunostained for VEGF-A. Only eleven meningiomas showed positive VEGF-A staining, and nine of these were positive for VEGFR-2. The PTBE was evaluated subjectively using Goldman's method (41). Souto et al. did not find any correlation between VEGF-A, VEGFR-2 and PTBE (45). The previously mentioned study by Otsuka et al. (39) also evaluated the VEGFR-1 and VEGFR-2 protein expression by immunohistochemistry. They noted whether tissue samples were positively or negatively stained for the VEGFR-1 and VEGFR-2, and found that the positively stained samples had significantly higher levels of VEGF-A as well as edema indices. Recently, Preusser et al. published a retrospective study of 8 recurrent, non-anaplastic meningiomas (46). They immunostained tissue sections for VEGF-A, and CD34, and performed in-situ hybridization for VEGF-A, VEGFR-1, and VEGFR-2 mRNA. The VEGF-A protein sections were categorized as positive/negative, cross-sectioned capillaries were counted in "hot-spots", and the VEGF-A, VEGFR-1, and VEGFR-2 mRNA sections were categorized into four levels of hybridization. VEGFR-1 and VEGFR-2 sections only showed hybridization in association with the endothelial cells. MIB-1 levels were significantly higher in samples with higher VEGF-A mRNA expression, and patients with capillary length longer than 552 mm/mm^3 had significantly higher recurrence rates. Our own studies showed that the VEGFR-2 mRNA levels were not increased in neither non-angiomatous nor angiomatous meningiomas compared to controls, but that the VEGFR-2 protein level in the angiomatous meningiomas was significantly increased compared to both non-

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[1] Schmid et al. calculated the edema index as PTBE/tumor size. We transformed the data so that it became comparable with our own edema index.

angiomaous meningiomas and controls (Table 2). This difference in VEGFR-2 protein level was independent of the longer capillary length of the angiomatous meningiomas, leading us to believe that an increase in VEGFR-2 protein levels may independently contribute to the increase in PTBE.

VEGF-A and neuropilin-1
In addition to the VEGFR-2 studies, we also found two studies by Barresi et al. from 2009 and 2010. In the older study, Barresi et al. (47), retrospectively chose 45 meningioma patients with different WHO grades, and investigated them for semaphorin-3A protein level and capillary length. They found that high semaphorin-3A levels were correlated to lower capillary length, and that a capillary length > 40 mm/mm^3 was a negative prognostic factor. In 2010 they expanded the study to include 48 patients (22), and semi-quantified VEGF-A protein and neuropilin-1 levels in addition to semaphorin-3A and capillary length. The study showed a positive correlation between semaphorin-3A and neuropilin-1 as well as VEGF-A and capillary length. In addition to the previous negative correlation between semaphorin-3A and the negative prognostic value of capillary length, Barresi et al. also found that a high VEGF-A/semaphorin-3A ratio was significantly correlated to a high histological grade and recurrence rate. In our own studies we found that neuropilin-1 protein was lower in angiomatous compared to non-angiomatous meningiomas and controls, as well as in non-angiomatous meningiomas compared to controls (Table 2). Because of the above findings, and the fact that semaphorin-3A bound to neuropilin-1 inhibits angiogenesis by inducing apoptosis in endothelial cells (21), we believe that neuropilin-1 has a dual role as both a VEGF-A agonist and an antagonist in conjunction with increased semaphorin-3A.

Sex-hormone receptors
Having found higher water content levels in male patients in paper I, we investigated the sex-hormone receptor expression in paper III. The estrogen receptor expression in the meningiomas was practically zero in our study. The immunostaining protocol for estrogen receptors is routinely performed at the Neuropathological laboratories, and we made sure to test the protocol on breast cancer cells. We are therefore confident in our results. Custer et al. found that 98% of the investigated 143 meningiomas in their study were negative for estrogen receptors (48). Like us, they used antibodies from DAKO. Brandis et al. investigated sex-hormone receptor levels in 60 meningioma patients in 1993 (49). Tumor size was measured for 43 patients on CT-scans, and PTBE was noted as either present or not-present on T2-weighted MRI. As with Custer's and our own results, Brandis et al. did not find any estrogen receptor expression in his dataset—they used antibodies supplied from Abbott Laboratories in Germany. The meningiomas were semi-quantified for progesterone receptor expression, but this parameter was not significantly correlated to PTBE or any other factor (49). Suoto et al. also investigated the progesterone receptor expression in their meningiomas samples, but did not find a correlation to PTBE (45). In 2004, Hasselblatt et al. investigated 38 angiomatous meningiomas from patients treated between 1983–1999 (50). The WHO-guidelines for diagnosis of angiomatous meningiomas were not followed, as grade II and III meningiomas with capillary indices above 50% were also categorized as angiomatous meningiomas. Pre-operative CT-scans were available for 35 patients, and these were categorized either with or without PTBE. Meningiomas were immunostained with progesterone receptor antibodies, and it was found that female

patients had higher progesterone receptor expression. Eventhough endothelial cells were immunostained, capillary length or microvascular density was not calculated. Other studies have found positive estrogen receptor indices in meningiomas, but did not investigate the PTBE, capillary length or the VEGF-A pathway (2,51). Based on the above findings, we do not believe that sex-hormone receptors are correlated to the PTBE in meningiomas.

METHOD COMPARISON
As part of our investigation of the VEGF-A pathway in meningiomas, we chose to implement a novel method for mRNA quantification. The new method (Lumistar) was based on chemiluminescence and selected because mRNA could be directly detected in the cell lysates. We also used the Lumistar assay as it could detect mRNA even in formalin-fixed paraffin-embedded tissue, although this capability was never needed. Prior to paper I, we ran a number of test-assays to ensure that the method was dependable, but failed to identify any independent studies on chemiluminescence. As the available studies did not compare the method directly to RT-qPCR, we chose to conducted a method comparison study ourselves. The first tests showed that in spite of the manufacturers statement, different incubation times resulted in varied results. Normalization by housekeeping genes is essential in mRNA quantification. In case of different incubation times, the signal decay of target mRNA and housekeeping gene must be quantifiably identical. Otherwise, the target-mRNA/housekeeping-gene ratio will differ in-between experiments. We found that this was not the case with Lumistar. Initially, the signal increased before starting to decay at different rates after 60 minutes, with sample-to-sample variance. We were not able to deduce the cause of the variability. Prolonging the incubation time would therefore be a source of further error. In case of instrument saturation, laborious re-sampling was preferred to prolonging the incubation. In papers I and III constant incubation times had been used, and re-sampling was performed as needed.

The classical method to investigate agreement between different methods is to use Bland-Altman (52) plots. This method is ideal when comparing different methods with the same output, e.g. blood pressure measurement were output is in mmHg. In the case of mRNA quantification, the output of RT-qPCR and Lumistar is different. RT-qPCR quantifies the amplified cDNA signal and presents this as a surrogate measure of the mRNA level. Lumistar quantifies amplified mRNA signal. The RT-qPCR data is exponential while the Lumistar output is linear. Bland-Altman's method could therefore not be used. In order to test the accuracy of the methods, dilution series with an arbitrary starting concentration were prepared. The RT-qPCR output (Ct) was transformed using the formula $2^{-Ct} \cdot 10^9$, and the transformed RT-qPCR and Lumistar values were compared to the dilution series. Both generated reliable, concentration-dependent data, although the Lumistar data was more consistent. We believe that particularly the steps requiring pipetting of minute amounts of sample material (1–2 μL) hold errors accounting for the lower accuracy.

As the housekeeping gene GAPDH is considered to remain constant in identical tissue types (32) we chose to normalize the RT-qPCR and Lumistar output using this housekeeping gene. As the GAPDH output introduced further variability, the R2 value between RT-qPCR and Lumistar VEGF-A/GAPDH ratios were lower compared to the accuracy shown in the dilution series. Yet, the result was still a significant linear correlation between RT-qPCR and Lumistar.

We found that Lumistar was competitive to RT-qPCR, and reflected a similar pattern in gene expression measurement with a similar accuracy, in spite of the vast difference of the molecular biological principles that the methods were based upon. The preferred method should therefore be chosen based on the variability of the samples, budget, and time at hand. When quantifying tissues without extreme variance in mRNA levels Lumistar can be superior to RT-qPCR as well as less time-consuming. It is also possible to quantify mRNA in a wider variety of tissue types compared to RT-qPCR. Still, RT-qPCR excels at quantification in tissues with a vast variance in mRNA expression, is more affordable, and enables the user with more control through application of homemade reagents.

PERSPECTIVES FOR OTHER FACTORS OF PTBE, FUTURE TREATMENT AND CLINICAL TRIALS
In recent years, many researchers have taken an interest in the aquaporin-family of transmembrane, passive, selective water-transport proteins in regards to water transport in the CNS. Aquaporin-1 and aquaporin-4 are found in the brain around the ventricles, where they probably have a function in the secretion of cerebrospinal-fluid from the arachnoid villi (53). Aquaporin-4 is also expressed in the glia limitans throughout the central nervous system (54). Several studies have shown that during ischemia, aquaporin-4 increases in peri-capillary astrocyte foot-processes, and that aquaporin-4 is increased in gliomas (53). Recently, Ng et al. found that aquaporin-4 was not restricted to the peri-microvascular region in 36 patients with intracranial meningioma (55). Ten of these patients had PTBE on MRI, and the aquaporin-4 protein level in their tissue samples was significantly increased. Wang et al. examined 59 patients with intracranial meningiomas visualized on MRI (56). The aquaporin-4 protein expression was quantified by western blotting, and aquaporin-4 and VEGF-A protein were semi-quantified by immunofluorescence histochemical staining. Aquaporin-4 was significantly increased in the group with PTBE, as was VEGF-A. Interestingly, aquaporin-4 and VEGF-A expression seemed to overlap on the immunofluorescence sections. Although the present material on aquaporin-4 in meningiomas with PTBE is limited, it does seem that aquaporin-4 has a role in PTBE formation alongside VEGF-A.

Matrix metalloproteinase 9 (MMP-9) is a peptidase shown to degrade the peritumoral extracellular matrix (23). It is believed that the degraded extracellular matrix eases migration of endothelial cells through the basement membrane into the peritumoral tissue. Several studies conducted in the last decade suggest that MMP-9 is a component in angiogenesis. Bergers et al. (57) showed that MMP-9 activated an angiogenic switch which induced angiogenesis through VEGF-A binding to VEGFR-2. In 2010, Ebrahem et al. found that MMP-9 facilitated VEGF-A secretion (58). Others have found a correlation between increased MMP-9, VEGF-A, and PTBE in meningioma patients (42,44,59,60). Nordqvist et al. found that meningioma patients with PTBE (N= 11) had significantly higher MMP-9 mRNA compared to those without PTBE (N = 5) (59). Among meningiomas with PTBE, those with larger PTBE also had higher MMP-9 (59). These results were confirmed by Paek et al. in 2002 and 2006, when they found a positive correlation between MMP-9 and PTBE in meningiomas (42,60).

In 2012, Iwado et al. investigated 60 meningioma patients for PTBE, VEGF-A and MMP-9. They found that VEGF-A and MMP-9 were significantly increased, and that meningiomas with high MMP-9 levels had a non-significant tendency for larger PTBE (44).

Other factors that may influence the angiogenesis and PTBE formation in meningiomas include the protein endothelin-1, which has been found to be associated with capillary length and VEGF-A (61), caveolin-1 which is also correlated to capillary length, and somatostatin which inhibits VEGF-A and angiogenesis (23).

The anti-VEGF drug bevacizumab (Avastin, Roche) is used as adjuvant chemotherapy of colorectal, breast, pulmonal, kidney, ovarian, and peritoneal cancers along with glioblastoma multiforme (62). Bevacizumab binds to VEGF-A and inhibits its binding to VEGFR-1 and VEGFR-2. Studies have shown that bevacizumab treatment of patients with glioblastoma multiforme decreases the PTBE (63,64). It is therefore plausible that bevacizumab can be used for therapy of meningiomas with PTBE. Sorafenib (Nexavar, Bayer) and sunitifib (Sutent, Pfizer) are VEGF-A receptor inhibitors, and are used as adjuvant chemotherapeutic agents against renal cell tumors and gastrointestinal-stroma tumors (1). A third receptor inhibitor, cediranib, has been shown to decrease glioblastoma multiforme PTBE, albeit only in an animal model (65).

7. CONCLUSIONS
We stipulate that meningiomas secrete VEGF-A which induces angiogenesis and tumor growth. This leads to decreased tumor blood flow and increased hypoxia. Due to the growth-induced hypoxia, VEGF-A and neuropilin-1 are up-regulated, which eventually leads to increased capillary length in order to reduce the hypoxia. Because of the increased capillary length, VEGFR-2 also increases, although the higher VEGFR-2 level cannot solely be explained by the longer capillary length. VEGFR-2 and neuropilin-1 bind VEGF-A and instigate further angiogenesis of capillaries with increased permeability, due to disruption of the normally expressed tight junction proteins through a currently unknown mechanism. The disrupted blood-brain-barrier results in an influx of plasma from the capillaries to the brain tissue. The elevated level of proteins in the extracellular matrix (ECM) of the brain increases the osmotic pressure gradient towards the ECM even further. If the function of the blood-brain-barrier is compromised because of other factors, e.g. due to the expression of aquaporin-4 on the endothelial cells, the increased osmotic pressure gradient will result in an additional influx of water through the passive aquaporin-4 water channels, ultimately resulting in massive PTBE. We hypothesize that this activation of the VEGF-A pathway first takes place inside the meningioma tissue, but with increased tumor water content and capillary expansion secretes into the peritumoral brain tissue. It is possible that if pial blood supply to the tumor is present, the PTBE formation will happen much more rapidly.

Neuropilin-1 plays a double role as both an agonist and an antagonist of angiogenesis in adjunction with semaphorin-3A. Because neuropilin-1 binds both VEGF-A and semaphorin-3A, the ratio between these two proteins either increases or decreases
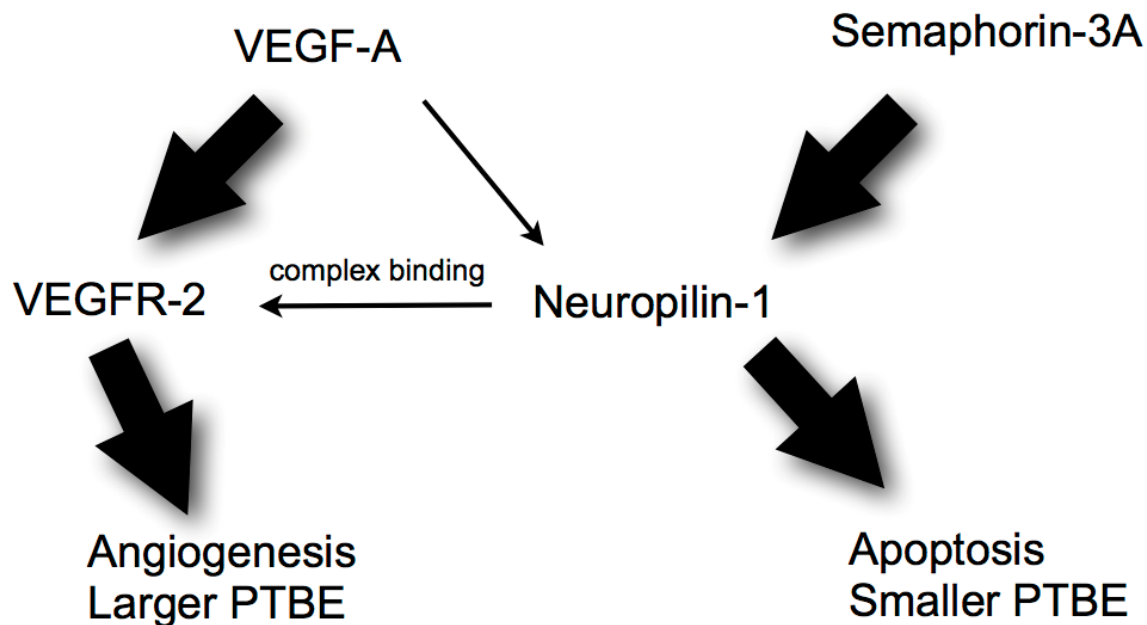
Figure 6
A flow chart showing the dual role of neuropilin-1 as an agonist of angiogenesis and possibly PTBE formation when in complex binding with VEGF-A, and as an antagonist when binding semaphorin-3A, in which case it induces endothelial cell apoptosis.

the PTBE-formation due to competitive inhibition (Fig. 6). In the pursuit of a medical treatment of meningiomas with PTBE, focus should be place on inhibitors of the VEGF-A pathway, in particular VEGF-A, as inhibitory binding of the VEGF-A protein itself will result in a decreased VEGF-A/semaphorin-3A ratio which will further the antagonistic effect on angiogenesis and PTBE formation. Since we believe VEGFR-2 also plays an independent role in the VEGF-A pathway, anti-VEGFR-2 might have a place as adjuvant treatment. Further investigation of the relationships between VEGF-A, semaphorin-3A, neuropilin-1, capillary length, and edema index in meningiomas as well as aquaporin-4, MMP-9, endothelin-1, caveolin-1, and somatostatin is needed in order to develop additional definite, non-surgical methods for the treatment of meningiomas with brain edema.

8. SUMMARY OF THE THESIS

Meningiomas are the second-most common intracranial tumors in adults. They are derived from the arachnoid cells, and although approximately 90% of meningiomas are benign, more than half of all meningiomas develop peritumoral brain edema (PTBE), which increases morbidity. The PTBE can be treated with steroid therapy, but this treatment is not specific, is not always effective, and involves long-term side-effects. Meningiomas are treated with radiation therapy, stereotactic radio-surgery or surgical resection. At the moment surgical resection is the only definite treatment, and the removal of the tumor also removes the PTBE. Based on the localization of the meningioma, surgery can be complicated. Although PTBE around meningiomas is frequent, the mechanisms behind its development are not clearly understood. It is believed that due to tumor growth and local tissue hypoxia, angiogenesis is increased and leads to the formation of PTBE. The angiogenic

protein vascular endothelial growth factor A (VEGF-A) is believed to be involved in the formation of PTBE around meningiomas, as several studies have found that it is increased in meningiomas with PTBE. VEGF-A is also known as vascular permeability factor due to its ability to increase the permeability of capillaries.

Paper I examines the VEGF-A protein and mRNA levels in 101 intracranial meningiomas. The PTBE is quantified on MRI, and capillary length and tumor water content are measured and compared to control brain tissue. Possible co-factors to PTBE like meningioma localization and subtypes are also examined. Forty-three of the patients have primary, solitary, supratentorial meningiomas with PTBE. The correlation between PTBE or edema index with the VEGF-A protein and mRNA, capillary length, and tumor water content is investigated in these patients. A novel method is used for mRNA quantification. It involves direct amplification of the mRNA with probes and branched DNA in order to produce a chemiluminescence signal that can be measured using a luminometer. The paper shows that the edema index is correlated to the VEGF-A protein and mRNA, and that capillary length is correlated to the PTBE. It also finds that VEGF-A protein and mRNA, capillary length and water content is increased in meningiomas compared to control tissue, suggesting that VEGF-A is produced in, and possibly secreted from the meningiomas. In addition, supratentorial meningiomas are shown to have larger PTBE compared to infratentorial meningiomas, suggesting that infratentorial meningiomas are diagnosed and removed earlier, due to earlier symptom development based on the anatomical features of the fossa posterior. Finally, a gender-specific difference in tumor water content and VEGF-A protein is revealed (higher and lower in females, respectively).

Paper II is a method-comparison study pitting the chemiluminescence assay against the often used quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) assay. In RT-qPCR, RNA is isolated, measured, reverse transcribed, purified, amplified via real-time PCR, and analyzed. The method is robust and reliable, albeit laborious to some extent. The chemiluminescence assay detects RNA directly without the need for RNA purification, complement DNA synthesis or cyclic amplification. By comparing the output of the two protocols to a dilution series ranging from 1 to 128 times of the homogenized samples, the precision of the protocols is measured. Furthermore, VEGF-A/GAPDH ratios are quantified for 15 tissue samples and the results compared between the two protocols, showing significant correlation. The study finds that the chemiluminescence assay is competitive to RT-qPCR, and reflects a similar pattern in gene expression measurement with a similar precision. Whether one method or the other should be used depends on the variability of the samples, budget, and time. RT-qPCR has a much wider dynamic range, and is preferable in case of significant sample inter-variability. It is also less expensive, and gives the user more flexibility as homemade reagents can be used. On the other hand, the chemiluminescence assay is straight forward, requires less hands-on-time, and can be used on formalin-fixed and paraffin-embedded (FFPE) tissue.

Paper III continues the investigations in paper I. The sample size is increased so that 22 angiomatous and secretory meningiomas are compared to 40 non-angiomatous meningiomas and 10 control brain tissue samples. Angiomatous and secretory meningiomas are chosen because they are known to have larger PTBE compared to other meningiomas. In addition to VEGF-A, capillary length, and PTBE, the VEGF-A tyrosine kinase receptor VEGFR-2 mRNA and protein levels are also examined. VEGFR-2 is a transmembrane receptor found on endothelial cells. It binds VEGF-A and thereby increases angiogenesis. VEGFR-2's co-receptor neuropilin-1 is also examined. Neuropilin-1 is an agonist of angiogenesis through complex-binding of VEGF-A, but it can also work as an inhibitor through competitive binding of semaphorin-3A. The complex binding of semaphorin-3A to neuropilin-1 can also induce endothelial cell apoptosis, thus working as an antagonist of angiogenesis. The study finds that VEGF-A mRNA, VEGF-A protein, and neuropilin-1 mRNA are higher in angiomatous and non-angiomatous meningiomas compared to controls. VEGFR-2 protein is higher, and neuropilin-1 protein lower in angiomatous meningiomas compared to controls. The mean capillary length is 3614 mm/mm^3 in angiomatous, 605 mm/mm^3 in non-angiomatous meningiomas, and 229 mm/mm^3 in the controls. Non-angiomatous and angiomatous meningioma patients have equally sized tumors. The mean PTBE around the angiomatous meningiomas is 695 cm^3, i.e. 477 cm^3 larger than the non-angiomatous meningiomas (p = 0.0045), and the mean edema index is twice the size compared to the non-angiomatous meningiomas. Further comparison between the two meningioma groups shows that mean VEGF-A mRNA, VEGFR-2 protein, and neuropilin-1 mRNA is significantly higher and neuropilin-1 protein is lower in the angiomatous meningiomas.

We believe that the VEGF-A pathway participates in the formation of PTBE in meningiomas by inducing formation of "leaky" capillaries, resulting in secretion of VEGF-A and plasma to the peritumoral brain tissue. It may therefore be worth pursuing therapies targeted directly against VEGF-A and its receptors through drugs like bevacizumab, sorafenib, sunitifib, and cediranib.

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