Measurement of \textit{in vivo} and \textit{in vitro} oxygen levels in the cerebrospinal fluid compartment during infection with \textit{Streptococcus suis}

THESIS
Submitted in partial fulfilment of the requirements for the degree
DOCTOR OF PHILOSOPHY (PhD)
awarded by the University of Veterinary Medicine Hannover

by

Alexander Martens (Lübeck)

Hannover, Germany 2019
Supervisor: Prof. Dr. Maren von Köckritz-Blickwede

Supervision Group: Prof. Dr. Maren von Köckritz-Blickwede
Prof. Dr. Peter Valentin-Weigand
Prof. Dr. Roland Nau

1st Evaluation: Prof. Dr. Maren von Köckritz-Blickwede
Institut für Physiologische Chemie,
Research Center for Emerging Infections and Zoonoses (RIZ),
Tierärztliche Hochschule Hannover

Prof. Dr. Peter Valentin-Weigand
Institut für Mikrobiologie,
Tierärztliche Hochschule Hannover

Prof. Dr. Roland Nau
Geriatrisches Zentrum,
Evangelisches Krankenhaus Göttingen-Weende

2nd Evaluation: Prof. Dr. Martin Diener
Institut für Veterinär-Physiologie und –Biochemie,
Justus-Liebig-Universität Gießen

Date of final exam: 01.11.2019

Sponsorship: Niedersachsen-Research Network on Neuroinfectiology (N-RENNT)
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List of Abbreviations

% percentage
BBB blood-brain-barrier
BCSFB blood-cerebrospinal fluid-barrier
BGA blood gas analysis
BLMB blood-leptomeningeal-barrier
BMEC brain microvascular endothelial cells
C celsius
CFU colony forming units
CNS central nervous system
CO₂ carbon dioxide
CPEC choroid plexus epithelial cells
CSF Cerebrospinal fluid
DAPI 4',6-diamidino-2-phenylindole
DLR Dual lifetime referencing
e.g. latein: exempli gratia (for example)
et al. latein: et alii
EU European Union
FACS fluorescence-activated cell scanning
fig figure
FTC flow through cell
h Hour
HIBCPP human choroid plexus papilloma
min Minute
ml Milliliter
NADPH nicotinamide adenine dinucleotide phosphate
NETs neutrophil extracellular traps
ns. non significant
O₂ oxygen
p.i. post infection
p.m. post mortem
paO₂ arterial oxygen pressure
pCSF cerebrospinal fluid pressure
pH power of hydrogen
PIM pimonidazole
PMN polymorphnuclear leukocytes
POF polymer optical fiber
RSP9 ribosomal protein S9
S. suis Streptococcus suis
sO₂ blood oxygen saturation
ST serotype
T Time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TEER</td>
<td>transepithelial electrical resistance</td>
</tr>
<tr>
<td>TJ</td>
<td>tight junction</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>ZO</td>
<td>zonula occludens</td>
</tr>
<tr>
<td>Δ</td>
<td>Delta</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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1. Summary (engl.)

Alexander Martens

**Measurement of in vivo and in vitro oxygen levels in the cerebrospinal fluid compartment during infection with Streptococcus suis**

Oxygen is an elementary factor in the metabolism of cells. At different oxygen concentrations, the organism reacts through a complex system of oxygen uptake, transport and delivery to the target organs, as well as numerous adjustments at the level of the individual cells. This leads to altered phenotypes and cell behavior.

To study various biological processes, e.g. the host-pathogen interactions in case of infection, cell cultures are often used. It should be the claim of science to adapt these cell cultures to the in vivo prevailing conditions as good as possible in order to generate transferable research results. In the past, experiments using cell cultures were often performed under standard incubator conditions, which usually corresponds to an oxygen content of 18-21% in the air. Thus, cell cultures need to be adapted to physiological relevant oxygen level.

The goal of this study was to characterize in vitro and in vivo oxygen level during Streptococcus (S.) suis meningitis. *S. suis* is a zoonotic bacterium that can infect humans and pigs. As a first goal, characterization of oxygen concentration (along with pH measurement) was performed on a cell model that mimics the blood-cerebrospinal fluid barrier (BCSFB). For this purpose, human choroid plexus epithelial cells (HIBCPP) were incubated on an inverse transwell filter system and were infected with *S. suis*. To mimic the interaction with immune cells, the model was supplemented with freshly harvested human neutrophil granulocytes that interact with the bacteria after transmigration of the cell barrier. The model showed oxygen partial pressures of about 22 mmHg corresponding to 3% oxygen after 6 hours of incubation with integrated *S. suis* infection and subsequent PMN interaction whereas the oxygen levels in uninfected HIBCPP dropped only to a minimum of 56 mmHg corresponding to 7-8% oxygen.
In order to compare these oxygen values found in the cell culture with the *in vivo* situation, as a second aim, corresponding measurements were carried out directly in the cerebrospinal fluid of infected pigs. The pigs were infected intravenously with *S. suis* and, after 13, 16 and 19 hours post infection the oxygen content (parallel to a pH determination) was measured *in vivo* in the CSF under controlled anaesthesia of the animals. Microbiological and histopathological examinations were performed on numerous target organs to characterize the course of infection. It was found that the oxygen levels in the CSF in healthy animals are also about 7-8%, which corresponds to the conditions found in the cell culture. However, a further drop in the oxygen levels in the context of infection and thus oxygen consuming, migrated bacteria and PMN in the CSF did not occur in those infected animals investigated here.

However, the discrepancy of the oxygen values under infection conditions between the cell culture and the *in vivo* situation should lead to an adaptation of the cell model, in order to better mimic the true pathophysiological situation.

An adaptation of the oxygen level in the HIBCPP cell culture can lead to a further development of this model that is used for the research of host-pathogen interactions and thus meet the claim of the so-called 3R principle to replace animal experiments by suitable alternative methods.
2. Zusammenfassung (dt.)

Alexander Martens

In vivo und in vitro Sauerstoffmessungen im Cerebrospinalflüssigkeitskompartment während einer Streptococcus suis - Infektion


Um verschiedene biologische Prozesse zu untersuchen, z.B. die Wirt-Erreger-Wechselwirkungen im Falle einer Infektion, werden häufig Zellkulturen eingesetzt. Es sollte der Anspruch der Wissenschaft sein, diese Zellkulturen so gut wie möglich an die in vivo herrschenden Bedingungen anzupassen, um übertragbare Forschungsergebnisse zu generieren. In der Vergangenheit wurden Experimente mit Zellkulturen häufig unter Standard-Inkubatorbedingungen durchgeführt, was normalerweise einem Sauerstoffgehalt von 18 - 21% in der Luft entspricht. Daher müssen Zellkulturen an den physiologisch relevanten Sauerstoffgehalt angepasst werden.


Als erstes Ziel wurde die Charakterisierung der Sauerstoffkonzentration (zusammen mit der pH-Messung) an einem Zellmodell durchgeführt, das die Blut-Liquor-Schranke (BCSFB) nachahmt. Zu diesem Zweck wurden humane Plexusepithelzellen (HIBCPP) auf einem inversen Transwell-Filtersystem inkubiert und mit S. suis infiziert. Um die Interaktion mit Immunzellen nachzuahmen, wurde das Modell mit frisch gewonnenen, menschlichen neutrophilen Granulozyten ergänzt, die nach der Transmigration der Zellbarriere mit den Bakterien interagieren. Das Modell zeigte Sauerstoffpartialdrücke von etwa 22 mmHg (entspricht ca. 3% Sauerstoff) nach 6-stündiger Inkubation mit integrierter S. suis-Infektion und anschließender
Granulozyten-Wechselwirkung, während der Sauerstoffgehalt bei nicht infizierten HIBCPP nur auf ein Minimum von 56 mmHg (entspricht ca. 7-8% Sauerstoff) abfiel.


Zur Charakterisierung des Infektionsverlaufs wurden an zahlreichen Zielorganen mikrobiologische und histopathologische Untersuchungen durchgeführt. Es wurde festgestellt, dass der Sauerstoffgehalt im Liquor bei gesunden Tieren ebenfalls etwa 7 bis 8% liegt, was den Bedingungen in der Zellkultur entspricht. Ein weiterer Abfall der Sauerstoffwerte im Rahmen der Infektion infolge eines Sauerstoffverbrauchs migrierter Bakterien und Neutrophiler Granulozyten im Liquor trat bei den hier untersuchten infizierten Tieren jedoch nicht auf.

Die Diskrepanz der Sauerstoffwerte unter Infektionsbedingungen zwischen der Zellkultur und der in vivo-Situation sollte zu einer Anpassung des Zellmodells führen, um die wahre pathophysiologische Situation besser nachzuahmen.

Eine Anpassung des Sauerstoffgehalts in der HIBCPP-Zellkultur kann zu einer Weiterentwicklung dieses Modells führen, das für die Erforschung von Wirt-Pathogen-Wechselwirkungen verwendet wird und somit den Anspruch des sogenannten 3R-Prinzips erfüllen, Tierversuche durch geeignete Alternativen zu ersetzen.
3. Introduction

Animal experiments still play an important role in science today. First reports on the use of animals in science date back to ancient Greece from the years 400 to 300 BC. Especially the polymaths Aristotle (384-322 BC) and Erasistratus (304-258 BC) documented animal experiments (Lucke 1986). The Roman physician Galenos of Pergamon (2nd century) is known to have made anatomical studies on animals, which earned him the name of "Father of Vivisection". The Arab physician Avenzoar practiced anatomical examinations mainly on deceased animals in order to test surgical practices for human use (Abdel-Halim 2005). Throughout history, animal experiments have steadily increased in popularity among researchers. However, they have also increasingly become the negative focus of the public and have become the subject of political discourse. Both the ethical responsibility of humans towards animals and the validity of transferring findings from animal experiments to human medicine are being reassessed. Consequently, the search for alternative methods for animal experiments has received a lot of attention in recent years. One manifestation of this was the consensus of the Member States of the European Union in 2010 to promote the development and validation of alternative methods in their countries (Directive 2010/63/EU of the European Parliament and of the Council of 22. September 2010 on the protection of animals used for scientific purposes). All EU member states now request scientists to monitor and document the number and species of animals used in their experiments. According to the German Federal Ministry of Food and Agriculture (BMEL), a total of 2,031,810 animals were used for scientific purposes in the year 2017 (excludes re-used animals). In addition, 738,484 animals were killed for scientific purposes without previous intervention or treatment (for example for the removal of organs or cell material). Mice, rats and rabbits make up the majority with 83.22 % of the laboratory animals (1,690,859 animals). Only 0.79% of the animals were pigs (16,130 animals). Of these, 2,664 were used in basic research and 8,817 in translational and applied research.

Alternative methods to animal experiments are all those methods, that can replace animal experiments, reduce the number of laboratory animals or reduce the suffering of laboratory animals. The scientific basis for the development of alternative methods is the so-called "3R principle", which was developed by William Russel and Rex Burch (Russell and Burch 1959).
Thereafter, an alternative method must reflect at least one of the following three requirements:

- **Replacement** (animal experiments will be replaced by non-animal methods)
- **Reduction** (the number of laboratory animals is reduced)
- **Refinement** (suffering or pain of the laboratory animals are diminished)

Nevertheless, there are discrepancies in the exact definitions and their implementation (Tannenbaum and Bennett 2015). Alternative methods include, for example, *in vitro* methods on cell cultures with isolated human or animal cells or computer simulations. However, the adaptation of cell cultures to *in vivo* relevant conditions is very difficult because of the variety of influences and the complex interaction of various factors (hormones, cytokines, interaction with other cells, pH, electrolytes, etc.). In the past, an often-overlooked player in cell culture research was the factor "oxygen" (Toussaint et al. 2011). Cell culture experiments usually still take place in "typical" cell incubators with humidified air and elevated CO₂ concentration, mostly at 5%. The oxygen content in the incubator thus corresponds to about 18-21%. It is important to note, that these oxygen levels are significantly higher than physiological oxygen levels that occur normally *in vivo* in different tissues.

Only in the last few years the factor oxygen has received more attention in basic cell science since unphysiological oxygen levels in *in vitro* experiments were shown to cause alterations in phenotypes and gene expressions (Wion et al. 2009; Kaneko and Takamatsu 2012; Branitzki-Heinemann et al. 2016), making the transfer of the results difficult to impossible compared to *in vivo* results.

In case of infection with a local high number of inflammatory cells and pathogens a lot of oxygen is consumed in the tissue. This "pathophysiological" condition often lowers the local oxygen availability to levels below normal and can be described as "true" hypoxia. Therefore, this is the condition that should be considered when infection experiments on cell cultures are performed.
The aim of this work is to determine the local oxygen concentrations occurring in the case of a \textit{S. suis} meningitis and to thereby shed more light on the basic physiological and physical laws concerning oxygen spread in the body \textit{(in vivo)} as well as in cell culture \textit{(in vitro)}. \textit{Streptococcus (S.) suis} is an important Gram-positive zoonotic pathogen which can spread from the blood to the brain via the blood-cerebrospinal-fluid-barrier (CSF)-barrier and lead to meningitis in humans and pigs. In this study, oxygen levels - together with pH level – are measured in an \textit{in vitro} choroid plexus epithelial model often used for meningitis research. The results were compared with \textit{in vivo} oxygen data obtained from animal experiments. For the latter, the oxygen - and pH - level were directly measured in the cerebrospinal fluid of living pigs infected with \textit{S. suis}.

The data of this work can provide the basis for optimizing this specific cell culture model for meningitis research regarding oxygen and pH. A later adaptation of the \textit{in vitro} model to the \textit{in vivo} relevant conditions can improve the transferability of scientific findings from the work on this cell culture model to the real-life situation. Consequently, it meets the requirements of the 3R principle of reducing or replacing animal testing by developing or optimizing substitute methods.

\textbf{3.1. The parameter “Oxygen”}

Oxygen is the most abundant element of the Earth's shell (49\%). It occurs in chemical compounds with other substances, as well as in the form of a covalent homodimer with the molecular formula \textit{O}_2 (dioxgen), called “molecular oxygen”. The melting point of oxygen is at 54.8 K (-218.3°C), its boiling point is at 90.15 K (-183°C) (Zhang, Evans, and Yang 2011). Under normal conditions (\textit{International Union of Pure and Applied Chemistry, IUPAC}: temperature of 273.15 K (0 °C) and absolute pressure of exactly 1 bar), molecular oxygen is a colour- and odourless gas contained in the air at 20.95\% (Dehnicke 1996). Oxygen is essential for all obligate aerobic eukaryotes (for example mammals) since it is needed for the respiratory chain in the mitochondria of the cells. Only in the 18th century oxygen was discovered as a separate substance. Independently of each other, the German-Swedish chemist Carl Wilhelm Scheele (1742-1786) and the English-American polymath Joseph Priestley (1733-1804) discovered oxygen during the investigation of combustion processes (Pilgrim 1951).
Oxygen is the only common gas that is attracted to the magnetic field. This means that it is paramagnetic and has unpaired electrons. Oxygen occurs in the basic form (so-called “triplet state”) and in excited states (so-called “singlet state”). In the low-energy basic form, the valence electrons are in a parallel spin expressed by the term symbol $3\Sigma_g^-$. However, the two excited states of oxygen, in which the valence electrons are aligned antiparallel and are either together in a $\pi^*$ orbital (terms symbol: $1\Delta_g$) or split into two $\pi^*$ orbitals (term symbol: $1\Sigma^+_g$), have significantly more energy. The latter state is energetically unfavourable and quickly changes to the $1\Delta_g$ state (Hasegawa et al. 2008). These properties of oxygen can be used to technically determine the concentration of oxygen molecules (see chapter 3.1.7.1).

### 3.1.1. Oxygen in the atmosphere and in cell incubators

Oxygen, together with nitrogen, forms the largest part of the breathing air. Far behind is argon, followed by various trace gases like CO$_2$. In dry air, molecular oxygen is present at approx. 20.95%, nitrogen at approx. 78.09%, argon at approx. 0.93% and CO$_2$ at approx. 0.039% (Raiswell 2007) (figure 1). These figures refer to dry air and do not consider the influence of water vapor (humidity), which varies with weather conditions and geographic situation. The relative humidity only describes the ratio of the absolute humidity and the maximum possible humidity at a given temperature and air pressure.

In a typical cell culture incubator, a water dish is usually added to increase the humidity in the incubator and thus counteract the evaporation of cell culture medium. In addition, the proportion of CO$_2$ in the cell incubator atmosphere is regularly increased to 5% to ensure a balanced pH (Williamson and Cox 1968; Schmitz 2011). The partial pressure of water vapor (pH$_2$O) at 37°C with 100 % humidity at sea level is specified as 47 mmHg (Ortiz-Prado et al. 2019; West 1993). This results in a percentage of 6.2% of the total air pressure at sea level ($p_{air}(0) = 760$ mmHg). Due to these additional gas partial pressures at constant total air pressure, the partial pressures of the other gases decrease. The proportion of oxygen in the total gas mixture is then only 18.6%. 
While in dry air, the oxygen content is 20.95%, this drops in standard cell incubators with increased humidity and up to 5% increased CO₂ level down to 18.6%.

3.1.2. Physical laws of gases

To understand the behaviour of oxygen and its solubility in liquids, some basic physical laws must be considered. The basic quantities for the analysis of gases are the substance quantity \( n \), the volume \( V \), the pressure \( p \) and the temperature \( T \). For some measurements also the salinity of a liquid can be of importance.

3.1.2.1. The Ideal Gas Law

The so-called Ideal Gas Law (Eq. 1.1) summarizes the findings of individual discoveries on the behaviour of gases. It was written in 1834 by Émile Clapeyron (1799 – 1864) and contains the discoveries and publications of Robert Boyle, Jacques Charles, Amedeo Avogadro and Joseph Louis Gay-Lussac and can be formulated as follows:

\[
\text{Eq. 1.1} \quad pV = nRT
\]

\( p \) = pressure; \( V \) = volume [L]; \( n \) = number of molecules of the gas [mol]; \( R \) = ideal gas constant (62.364 L mmHg K\(^{-1}\) mol\(^{-1}\)); \( T \) = temperature [K]

In 1661, the English physicist Robert Boyle (1627-1691) found out that the volume of a gas is anti-proportional to its pressure. Equation 1.2 shows the relationship between the volume and the pressure in case of constant \( n \) and \( T \).

\[
\text{Eq. 1.2} \quad V \sim \frac{1}{p} \quad \text{or} \quad pV = \text{const.} \quad \text{(Boyle's law)}
\]
How gases tend to expand when they are heated was published first by Joseph Gay-Lussac in the year 1802 but credited to the French scientist Jacques Alexandre César Charles (1746 – 1823) who made the investigation. Equation 1.3 shows the relationship.

Eq. 1.3 \[ V \sim T \] (Charles’s law)

In 1811, the Italian scientist Amedeo Avogadro (1776 – 1856) found out that equal volumes of any ideal gases have the same number of molecules when they are in the same temperature and under the same pressure (Eq. 1.4). If temperature and pressure are constant the number of molecules of the gas and its volume are proportional.

Eq. 1.4 \[ V \sim n \] (Avogadro’s law)

The chemist Joseph Louis Gay-Lussac (1778-1858) published in 1808 his discovery that the pressure of a known mass of gas varies directly with the absolute temperature of the gas, when the volume is kept constant. Equation 1.5 shows the so-called Gay-Lussac’s law.

Eq. 1.5 \[ \frac{p}{T} = \text{const}. \] (Gay-Lussac’s law)

3.1.2.2. *Dalton’s law of partial pressures*

Partial pressure is the pressure exerted by a single component or fraction in an (ideal) gas mixture. The partial pressure corresponds to the pressure a single gas component would exert if it were solely present in the respective volume. The total pressure is composed of the partial pressures of the gas components (figure 2). These relationships were discovered in 1805 by the English naturalist John Dalton (1766-1844) and described by the following formula, known as the 1st Dalton law of partial pressure (Eq. 1.6):

Eq. 1.6 \[ p_{\text{total}} = \sum_{i=1}^{n} p_{i} \]
The total pressure exerted by a gas mixture in a certain volume is composed of the individual components of the mixture \((p_A + p_B)\). Under standard conditions according to the International Union of Pure and Applied Chemistry (IUPAC), the total pressure of dry air is 760 mmHg at sea level at 0°C and is formed by the main partial pressures of nitrogen (~593.50 mmHg), oxygen (~160.00 mmHg), argon (~7.70 mmHg) and CO\(_2\) (~0.3 mmHg).

3.1.2.3. **Henry’s law**

The solubility of a gas in liquids is described by the so-called Henry’s Law (Eq. 1.7). This mathematical description states that the partial pressure of a gas \((P_g: \text{mmHg})\) above a liquid is directly proportional to the molar concentration of the dissolved gas \((C_d: \text{mM})\) in the liquid. The proportionality is expressed by the Henry constant \((H: \text{mmHg/mM})\).

\[
C_d = \frac{P_g}{H}
\]

The Henry constant for a liquid medium depends on its concentration of electrolytes, proteins and the actual temperature. Therefore, salt concentrations in liquids have to be considered when calculating oxygen amounts. In typical cell culture media however, the salt concentration is very low and can be neglected, whereas in seawater with higher salinity a reduced solubility of oxygen can be observed. The greater the Henry constant for a medium, the slower the gas dissolves in the liquid.

For cell culture media with average 175 mM electrolytes, a temperature of 37 °C and a 100 % humidity in the incubator (without increased CO\(_2\) concentration), a Henry’s constant of 771.65 mmHg / mM is calculated (Place, Domann, and Case 2017). Using this value of Henry constant an oxygen concentration of about 0.183 mol/L is found in a standard cell culture medium in a
cell incubator with increased CO₂ (5%), 100 % humidity and 37°C. This concentration is about 40 times lower than for the molar concentration of gaseous oxygen in the overlaying air. Due to the temperature dependence of the Henry constant, the solubility of gases in liquids can vary. As the temperature increases, the solubility is lower, meaning that gases leave the liquid. Vice versa, dropping the temperature leads to more dissolved gases in the aqueous medium (Weiss 1970).

3.1.2.4. Fick’s First Law

The Fick’s law of diffusion (Eq. 1.8) states that the diffusion rate or flow rate (F) of a gas through a medium is proportional to the difference of the gas concentrations (ΔC) of both sides and behaves inversely proportional to the thickness of the medium (Δx; diffusion length). The different solubilities of gases in different media are corrected by the Fick’s diffusion constant (D). There is hardly any information in the literature about diffusion constants for different cell culture media. Based on information from Goldstick et al. a Fick’s diffusion constant for cell culture medium with 10% fetal calf serum addition was calculated to be D = 2.84x10⁻⁵ cm²/s at 37 °C (Goldstick, Ciuryla, and Zuckerman 1976; Place, Domann, and Case 2017).

Eq. 1.8

\[ F = \frac{D \cdot \Delta C}{\Delta x} \]

The Fick's law of diffusion can, for example, be used to describe the oxygen accession from the alveoli in the lung into the bloodstream. This mechanism is comparable to that in a cell culture (figure 3).
A concentration gradient ($\Delta C$) between two compartments drives gases to diffuse. The resulting diffusion rate is inversely proportional to the length of the diffusion path ($\Delta x$). This principle occurs in the diffusion of oxygen from the alveoli into the blood ($\Delta x =$ alveolar membrane), as well as in the transfer of oxygen to the cells in a cell culture ($\Delta x =$ height of the medium).

The concentration gradients ($\Delta C$) between alveoli and blood or between cell incubator atmosphere and the cells of the cell culture are the driving force of diffusion. The concentration gradients extend over the thickness of the alveolar membrane or over the "thickness" of the medium above the cells in cell cultures. Figure 3 shows this schematically using an example of a cell culture model with human choroid plexus epithelial cells (HIBCPP) on an inverted Transwell Filter system (chapter 3.3). The "thickness" that oxygen molecules need to pass to arrive at the location of oxygen demand is an important factor.

### 3.1.2.5. Diffusion of oxygen from the air into the medium

The driving force for the phenomenon of diffusion of the oxygen molecules from the air into the blood or cell culture medium is a concentration gradient of the molecules between the air-media interface. If the partial pressures (not the concentrations) of the oxygen in both phases (gaseous vs. dissolved) are the same, this is called “equilibrium” (Pittman 2011).

Due to thermodynamic events however, molecules are released from the liquid and go back into the gas phase, but at the same time gas molecules also dissolve back into the liquid. The equilibrium state is maintained.

If oxygen is consumed by cells or other consumers (e.g. bacteria or parasites), the local amount of oxygen drops decreasing the local oxygen concentration. To restore the equilibrium, the oxygen molecules move in the direction of the reduced oxygen content. In order to keep the balance between the atmosphere and the liquid, more oxygen must dissolve accordingly. As
mentioned before, this process is governed by various influences (see chapter 3.1.2). If the loss of oxygen in the medium cannot be compensated, the overall oxygen content in the medium drops.

3.1.3. Different units for indicating amounts of oxygen

The amount and content of oxygen in the air or dissolved in liquids can be stated in several ways. The information is given either as a concentration - i.e., a relation to a theoretical absolute saturation of the air or a liquid (% saturation) in a defined environment - or as a partial pressure. Often, the volume/volume ratio of oxygen in the carrier medium (air or liquid) is also given in percentage. For the exact indication however, the percentage unit is only moderately suitable as it depends on the actual air pressure which, in turn, is influenced by geographical differences in altitude and subject to seasonal and daily changes. Table 1 illustrates the differences in the partial pressure (approximations) of oxygen despite a constant volume/volume (v/v) ratio of “normoxic” 20.95% (see chapter 3.1.1).

<table>
<thead>
<tr>
<th>attitude</th>
<th>barometric pressure [mmHg]</th>
<th>pO₂ [mmHg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead Sea (-425 m)</td>
<td>802.58</td>
<td>168.14</td>
</tr>
<tr>
<td>Sea level (0 m)</td>
<td>760.00</td>
<td>160.00</td>
</tr>
<tr>
<td>University of Veterinary Medicine, Hannover (65 m)</td>
<td>755.25</td>
<td>158.25</td>
</tr>
<tr>
<td>Denver University, Denver USA (1600 m)</td>
<td>630.80</td>
<td>132.15</td>
</tr>
<tr>
<td>Mount Everest (8848 m)</td>
<td>244.05</td>
<td>51.13</td>
</tr>
</tbody>
</table>

Table 1 Partial pressure of oxygen (pO₂) in dry air at different attitudes

Common units for the oxygen concentration are e.g. mol/L (mM), mg/L, ml/L or ppm. For the pressure indications usually the SI-unit Pascale (Pa) is used. This unit is defined as:

\[ 1 \text{ Pa} = 1 \frac{N}{m^2} = 1 \frac{kg}{m \cdot s^2} \]

Further often used units are bar, atmosphere (atm) and mmHg (torr). Table 2 shows factors for conversion into the different units. Dry air has 100% oxygen saturation under standard conditions as well as well-ventilated water in free exchange with the ambient air. According to the International Union of Pure and Applied Chemistry (IUPAC), the following conditions
apply as standard: temperature = 273.15 K (0°C); pressure = 1013.25 hPa (760 mmHg), no humidity.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Pa</th>
<th>bar</th>
<th>atm</th>
<th>mmHg / Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa</td>
<td>1</td>
<td>1.0000</td>
<td>9.8692</td>
<td>7.5006</td>
</tr>
<tr>
<td>bar</td>
<td>1.0000</td>
<td>1</td>
<td>9.8692</td>
<td>7.5006</td>
</tr>
<tr>
<td>atm</td>
<td>1.0133</td>
<td>1.0133</td>
<td>1</td>
<td>7.6000</td>
</tr>
<tr>
<td>mmHg / Torr</td>
<td>1.3332</td>
<td>1.3332</td>
<td>1.3158</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2 Conversion factors to calculate different pressure units

In addition to the arterial partial pressure (paO$_2$ in mmHg), which describes the proportion of dissolved oxygen in the arterial blood, there are further units for the description of oxygen conditions in the blood. Another unit associated with oxygen is the so-called “Oxygen saturation (sO$_2$)”. It indicates the percentage of oxygenated haemoglobin (see chapter 3.1.4) in the blood and thus allows to assess the functionality of the lung and the effectiveness of oxygen transport in the blood. Various designations are used to specify the sO$_2$-measurement. Thus, arterial saturation is abbreviated to SaO$_2$ (invasive measurement), a measurement via the skin using a pulse oximeter is abbreviated to SpO$_2$ and a venous saturation (invasive) to SvO$_2$.

Neither paO$_2$ nor sO$_2$ indicate the total concentration of oxygen in the (arterial) blood. For this the "Oxygen Content" (CaO$_2$) is used. The total concentration of oxygen (dissolved and bound to haemoglobin) is described by equation 1.9 (applies to humans):

Eq. 1.9 \[ C_{a}O_2 \left[ \frac{ml}{dl} \right] = S_{a}O_2 \left[ \% \right] \times Hb \left[ \frac{g}{dl} \right] \times 1.34 + p_{a}O_2 \left[ mmHg \right] \times 0.0031 \]

3.1.4. Oxygen transport via the blood

Each cell in the organism relies on the supply of sufficient dissolved oxygen. Oxygenation of tissues is one of the most important processes that occur within the body. Without proper oxygenation of tissues, metabolic processes cannot function efficiently, and cellular functions will be interrupted (Sharma and Rawat 2019). The carrier medium is the blood that flows through the body due to the circulatory system and transports the oxygen molecules, absorbed by the respiratory system, to the location of use. However, only a maximum of 2 %
of the oxygen in the blood is dissolved (von Engelhardt et al. 2015). To compensate for the poor solubility of oxygen in the blood, there is a highly complex "oxygen transport system" in the organism, namely the binding to haemoglobin, the main protein in red blood cells. According to the theory of evolution, it took millions of years for the formation of this transport system to form larger and more complex organisms. Previously, the organisms were therefore severely limited in size because oxygen diffused passively to the cells in the organism (Knoll 2011).

Via parallel arteries and arterioles, the oxygen is brought now very close to the cells via a complex network of capillaries. For most tissues, the diffusion distance is between 10-30 μm (Kety 1951). In a few cases, the distance is slightly larger, but is still usually less than 100 microns (Krogh 1919).

Ninety eight percent of the oxygen molecules are transported through the body bound to haemoglobin. An erythrocyte contains approximately 3x10⁸ haemoglobin molecules in its cytosol. Together with the enormous amount of red blood cells in the body of about 25 trillion (adult with about 80 kg body weight), the capacity of this transport system is exemplified (Horn 2012).

Haemoglobin is a spherical molecule consisting of four subunits. These in turn are composed of a porphyrin part (heme) and a protein part (globin), formed by four different polypeptide chains (α-, β-, γ- and δ-chain), that characterize the haemoglobin. The heme part undergoes chelation with divalent iron (Fe²⁺), each of which able to bind an oxygen molecule (figure 4). In total, this allows an entire haemoglobin molecule to take up four oxygen molecules.

The uptake of oxygen is reversible. Haemoglobin is an allosteric protein that alters its conformation when it binds to an oxygen molecule, thereby increasing its affinity for other oxygen molecules (cooperative interaction). Plotting the saturation against partial pressure results in a sigmoidal course (figure 5). A right shift can, for example, be caused by an increased CO₂ partial pressure, as it occurs in the tissue due to cell metabolism. The same effect can be observed following a temperature increase or an increased production of 2,3-bisphosphoglycerate (2,3-BPG), a substance produced by the body to adapt to different oxygen conditions, e.g. in case of a long stay at high altitudes with
lower oxygen partial pressure. Moreover, the right shift is influenced by a drop in pH. A right shift causes a decrease in affinity for oxygen (state in the tissue). A change of this factors in the opposite direction leads to a left shift and thus to an increase in affinity (condition in the lungs). The change in the affinity of haemoglobin for oxygen is called “Bohr effect”.

![Binding curve of oxygen to haemoglobin](image)

Figure 5  Binding curve of oxygen to haemoglobin
Due to the cooperative character of haemoglobin, the binding curve shows a sigmoidal course. Various factors affect the affinity and can shift the curve to the left or right.

3.1.5. Oxygen metabolism in cells and adaptations to hypoxia

Every cell in the body requires oxygen to produce Adenosine triphosphate (ATP), the cell’s “currency of energy”. Adult humans metabolize about 200 grams of oxygen per day (X. D. Wang and Wolfbeis 2014). Therefore, oxygen molecules diffuse passively into the cells. The driving force is the difference in concentration of oxygen between the intracellular and extracellular milieu. For fat-soluble substances such as oxygen molecules, the entire cell membrane of the consumer cell is available for this mechanism (Pittman 2011). The oxygen molecules are either dissolved directly in the blood stream or are bound to haemoglobin, from where it dissolves at the place of consumption due to the Bohr effect.

A simplified schematic of the following procedures according to Alberts et al., 2017 is shown in figure 6.
Through the blood plasma and the intercellular fluid, the oxygen molecules diffuse into the cytosol of the cells. From there, they enter the mitochondria, where they serve as maintainers of the so-called respiratory or electron transport chain and are "consumed".

Pyruvate and fatty acids from the cytosol are degraded in the mitochondria to Acetyl-CoA. The Acetyl-CoA is then metabolized in the so-called Citric acid cycle, which reduces Nicotinamide adenine dinucleotide (NAD') to NADH (or Flavin adenine dinucleotide (FAD) to FADH$_2$; not shown). By means of the so-called oxidative phosphorylation energy-rich electrons from NADH (or FADH$_2$) are then transported to the direction of oxygen along the electron transport chain in the inner membrane of the mitochondria. This electron relocation produces a proton gradient that is used to power the formation of ATP by the ATP synthase, using Adenosine diphosphate (ADP) and phosphate. Along the electron transport chain, the electrons pass through different protein complexes, with Complex I absorbing the electrons of NADH and Complex II absorbing electrons coming from FADH$_2$. Complex II, unlike Complex I, does not pump protons into the intermembrane space. The lipophilic molecule Q$_{10}$ (Ubiquinone) transfers the electrons to Complex III, which in turn serves as a proton pump. The protein Cytochrome-C finally transports the electrons from Complex III to Complex IV, in which the so-called Cytochrome-C oxidase catalyses the transfer of electrons to the molecular oxygen and thus reduces it to water (H$_2$O). Molecular oxygen is thus an essential reagent in the respiratory chain.
Oxygen molecules in the respiratory chain within the mitochondria

From different sources Acetyl-CoA is synthesized inside the mitochondrion. Acetyl-CoA is converted to NADH in the citric acid cycle by which electrons are released. These electrons transpass four complexes (I-IV) located on the inner membrane where H\(^+\)-ions are released simultaneously. The free H\(^+\)-ions pass through the intermembrane space to the ATP synthase, which transfers externally derived phosphate to ADP, thereby producing ATP, the cell's energy currency. To maintain the flow of electrons in the complexes, oxygen ultimately serves as an electron acceptor and is converted to water.

If there is an undersupply of oxygen, the ATP synthesis can be ensured shortly via the anaerobic degradation of glucose to lactate. However, the amount of ATP gained thereby is only about 5% of the amount that is otherwise obtained via the oxidative ATP synthesis (G. L. Semenza 2009; Mollenhauer and Kiss 2010). In addition to this lack of energy, there is also the formation of large amounts of cell toxic reactive oxygen species (ROS), because electrons are then transferred to elemental oxygen (J. Kim et al. 2006). The effect of the ROS can lead to irreversible cell damage, that can be seen sometimes in organ transplantations during inadequate oxygen supply ex vivo (Schmidt et al. 2008). Mammals, however, have a high adaptability to extreme oxygen conditions (e.g. high flying birds or deep diving whales) (Ramirez, Folkow, and Blix 2007).

An important role in the adaptation of the cells to decreasing oxygen concentrations is played by a very prominent transcription factor, the so-called Hypoxia-inducible factors (HIF) (Michael Swindle and Smith 2008). These factors consist of an α- and a β- subunit. The latter...
is constitutively expressed by the cells, while the α-subunit is subject to oxygen regulation (G. L. Wang et al. 1995). If enough oxygen is present, the HIF prolyl hydroxylases (PHD 1-3) will hydroxylate this subunit (Domann and Place 2013) and then bind it to von Hippel-Lindau tumour suppressor protein (pVHL) (Maxwell et al. 1999), ultimately proteasomally degrading the HIF α-subunit (Forsythe et al. 1996; Bruick 2001). If the α-subunits are not degraded by the PHD in the absence of oxygen, they bind to the β-subunit and are transported into the cell nucleus. There, HIF binds to the so-called hypoxia-responsive elements (HRE) in promoters of target genes whose transcription causes various adaptations to oxygen deficiency like the Vascular endothelial growth factor VEGF, which leads to an angiogenesis around the hypoxic cells and thus promotes an increased blood supply (Mohamed et al. 2004; Shweiki et al. 1992; Gregg L. Semenza 2014; Brahim-Horn, Chiche, and Pouysségur 2007; Goto et al. 1993; Prior, Yang, and Terjung 2004). In addition an increased formation of erythropoietin can be seen (G. L. Semenza 2009). In hibernating animals with reduced respiration the presence of HIF leads to a decrease of high oxygen-consuming metabolic processes (Andrews 2004).

How cells respond to hypoxia is influenced by their oxygen consumption rate (OCR), which has been studied for some cell lines (Wagner, Venkataraman, and Buettner 2011b). Cells with a high metabolism, such as hepatocytes, have a very high OCR between 200-400 attomoles (amol)/cell/s (Metzen et al. 1995). Other cell lines show OCR between 1 - 120 amol/cell/s (Wagner, Venkataraman, and Buettner 2011b). Interestingly, the HIF-PHD system reacts much earlier to decreasing O₂ partial pressure than it would be needed to maintain the function of cytochrome-C oxidase. The pO₂ or the oxygen concentration when the rate of cytochrome-C oxidase activity is ½ (P50/KM) is about 0.075-0.75 mmHg respectively 0.0097-0.097 mol/L (Scandurra and Gnaiger 2010). KM values for the HIF-PHD system are between 0.100-0.240 mol/L (Ehrismann et al. 2007; Hirsilä et al. 2003). This corresponds to an oxygen partial pressure between 70 and 75 mmHg (Place, Domann, and Case 2017).

3.1.6. Normoxia, physioxia and hypoxia

The oxygen environment of cells is often characterized by the terms “normoxia” and “hypoxia”. However, this can lead to misunderstandings, as normoxia is often used to describe the oxygen level of the room air or the air in the cell incubator (~ 18-21% O₂) and not only the physiological condition in the body for different cell types (physiological microenvironment). Therefore, the term “physioxia” is recommended when referring to this microenvironment.
Carreau et al compiled physiological oxygen values for various tissue types, other objects and air that are shown in table 3 (Carreau et al. 2011).

<table>
<thead>
<tr>
<th></th>
<th>$pO_2$ [mmHg]</th>
<th>$O_2$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>160</td>
<td>21.1</td>
</tr>
<tr>
<td>Inspired air (in the tracheus)</td>
<td>150</td>
<td>19.7</td>
</tr>
<tr>
<td>Air in the alveoli</td>
<td>110</td>
<td>14.5</td>
</tr>
<tr>
<td>Arterial blood</td>
<td>100</td>
<td>13.2</td>
</tr>
<tr>
<td>Venous blood</td>
<td>40</td>
<td>5.3</td>
</tr>
<tr>
<td>Cell</td>
<td>9.9–19</td>
<td>1.3–2.5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>&lt;9.9</td>
<td>&lt;1.3</td>
</tr>
<tr>
<td>Brain</td>
<td>33.8 ± 2.6</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>42.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Skin (sub-papillary plexus)</td>
<td>35.2 ±8</td>
<td>4.6 ±1.1</td>
</tr>
<tr>
<td>Skin (dermal papillae)</td>
<td>24 ± 6.4</td>
<td>3.2 ±0.8</td>
</tr>
<tr>
<td>Skin (superficial region)</td>
<td>8 ± 3.2</td>
<td>1.1 ±0.4</td>
</tr>
<tr>
<td>Intestinal tissue</td>
<td>57.6 ±2.3</td>
<td>7.6 ±0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>40.6 ± 5.4</td>
<td>5.4 ±0.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>72 ± 20</td>
<td>9.5 ± 2.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>29.2 ±1.8</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>48.9 ±4.5</td>
<td>6.4 ± 0.6</td>
</tr>
</tbody>
</table>

Table 3 **Oxygen level in different tissues** (Carreau et al. 2011)

Oxygen values below these physioxic levels can therefore be described as “hypoxia”. Alternatively, hypoxia can also be defined as state where oxygen delivery is no longer sufficient to maintain ATP production via oxidative phosphorylation (Gutierrez 1991). The term “anoxia” means a total depletion of oxygen ($pO_2 = 0$ mmHg) whereas an oxygen atmosphere that is too high compared to the physiologically normal situation is called “hyperoxia”. It was shown that oxygen atmospheres deviating from the physiological norm can have a negative influence on the cells and change their metabolism or their properties (Branitzki-Heinemann et al. 2016; Kaneko and Takamatsu 2012; Wion et al. 2009).

### 3.1.7. Measurement of oxygen

In order to describe the oxygen profile and the oxygen concentrations, there are various possibilities that are based on amperometry, photochemical or histological techniques. Clark et al. introduced in 1953 a method to measure oxygen in aqueous solutions (Clark et al. 1953),
that was optimized and adapted and is still used today (X. D. Wang and Wolfbeis 2014). The so-called Clark electrode is based on the principle of oxygen reduction at a cathode and oxidations on an anode leading to an electric current. Since the electrodes are separated from the measuring liquid by an oxygen-permeable membrane, the oxygen molecules diffuse correspondingly in proportion to their partial pressure to the electrodes. From this, the partial pressure of the oxygen can be calculated considering the influence of temperature. Clark separated the probes and the electrodes by cellophane, whereas teflon is usually used today. For the electrodes, platinum, silver, gold or lead are used. A disadvantage of the Clark electrode, however, is that it is an invasive measurement with a corresponding risk of contamination and the electrode itself influences the result by the consumption of molecular oxygen (Amao 2003).

For the detection of hypoxia in the tissue immunohistochemical staining with pimonidazole (PIM) (figure 7) can be performed. In hypoxic tissues, pimonidazole is activated and forms stable compounds with thiol groups of proteins, peptides and amino acids (Kutluk Cenik et al. 2013; Arnold et al. 2010; Varia et al. 1998). PIM belongs to the group of 2-nitroimidazoles and was formerly used as a supporting agent in radiotherapy. At an O₂ partial pressure of less than 10 mmHg, PIM is reduced by the enzyme 2-nitro reductase and binds to the free thiol groups within the cell (Franko and Chapman 1982; Raleigh et al. 1985). These PIM-thiol compounds can be afterwards labeled immunohistologically and displayed by using specific anti-PIM antibodies. However, negative immunohistologic results in PIM staining do not really exclude hypoxia (Jankovic et al. 2006), as hypoxia is tissue specific and can also occur with oxygen partial pressures greater than 10 mmHg (see chapter 3.1.6). Nevertheless, the amount of pimonidazole binding is proportional to the degree of hypoxia. An advantage of this method is the possibility to microscopically examine hypoxic areas in the tissue. Gradations in the degree of hypoxia can be detected but the actual prevailing oxygen conditions themselves are not determined.

For this reason luminescence-based measuring systems, also called “optodes”, have gained considerable attention in recent years and represent a good alternative method for
determining oxygen (Quaranta, Borisov, and Klimant 2012). Optodes can be used to determine oxygen conditions in vitro and in vivo. Their main advantage is the ability to measure current prevailing oxygen levels over longer periods of time. There are several manufacturers on the market, so that different systems can be offered and the technology is constantly evolving. Wolfbeis lists some advantages of luminescence-based measurements (Wolfbeis 2015):

- O₂ is not consumed during measurements
- Luminescence-based optical sensors are fully reversible
- Sensors can be designed for different levels of O₂ from very low to very high concentrations
- Remote sensing is enabled by using optical fibers
- A sterilized sensor layer can be placed in a sample and remotely and non-invasively read
- Planar sensors or nanosensors can be applied to imaging of O₂
- Multiple sensing at the same site is enabled by combining sensors for O₂ and other gases
- Targeted sensing is enabled by using nanoparticles that can recognize their target
- Sensors work equally well for even extremely dry gases and dissolved oxygen
- Optical sensors work well even in strong electromagnetic fields, in radioactive environments, and under hostile environmental or chemical conditions.

On the other hand, he advises to consider the following:

- Sensor membranes usually are made from materials that have high solubility for oxygen. Hence, they will extract O₂ from the sample which may lead to erroneous results in case of small sample volumes and/or very low levels of O₂.

However, the oxygen incorporation into the polymer matrix in optodes and thus apparent "consumption" is significantly lower than the active conversion of oxygen in measurements with the Clark electrode.
3.1.7.1. **Luminescence based measurements**

As described in chapter 3.1, oxygen has the property to exist in its basic form \( \text{O}_2 (^3\text{O}_2) \) and in an excited form \( \text{O}_2 (^1\text{O}_2) \). This property makes the molecule an excellent quencher for luminescence phenomena. All commercial solid-state optical sensors for \( \text{O}_2 \) rely on the use of luminescent probes (in a polymer matrix) whose emission is dynamically (by collision) quenched by triplet \( \text{O}_2 \) (Wolfbeis 2015). In detail (figure 8), this means that an LED with light of a specific wavelength irradiates a so-called fluorophore (indicator). Due to the absorption of the photons, the fluorophore enters a more energetic, excited state (1), in which there is a shift of an electron into higher energetic orbitals while maintaining the spin state \( S_0 \rightarrow S_1 \). Within the new energy level, some of the energy can be released by oscillation relaxation (2, internal transformation). In addition, the molecule can return to the ground state by the emission of photons (3, fluorescence). According to the rules of quantum mechanics, the fluorescence lifetime is short because the light emission is "spin allowed" and takes about \( 10^{-9} \) to \( 10^{-7} \) seconds (Lakowicz 2006; Puschnig 2013). Through the so-called intersystem crossing (4) the molecule changes its multiplicity by reversing the spin and changes into the so-called triplet state. The fluorophores used for the oxygen measurement have overlapping energy levels of the first excited singlet and triplet states, therefore it is highly probable that the intersystem crossing occurs in the presence of oxygen molecules. From here, the molecule can also fall back to its ground state, turning over its total spin. The energy is released as phosphorescence over a special time of decay (5). Oxygen molecules collide correspondingly to their amount in the sample with the fluorophore and take over a part of the luminescence energy. The triplet oxygen molecules therefore act as quenchers and become singlet oxygen molecules (6) (Friedmann, 2015).
Figure 8 Principle of luminescence quenching by oxygen molecules

After absorption of a photon’s energy the fluorophore enters a more energetic state while maintaining the spin state ($S_1$) (1). Some of the energy can be released due to internal transformations by oscillation relaxation (2). By the emission of photons, the molecule can fall back to its former state by generating fluorescence (3). By intersystem crossing the molecule changes from the singlet state to the triplet state by a spin reversal (4). From here the molecule can fall back to its ground state by the emission of phosphorescence (5). This phosphorescence can be changed by interaction with free oxygen molecules (6). Due to this interaction the amount of oxygen molecules can be determined by optode systems.

The more quencher molecules are in the sample, the more they decrease the phosphorescence signal of the indicator. This phenomenon is described by the *Stern-Volmer relationship* (Eq. 1.10) that describes the dependency of the luminescence intensity of a dye on the concentration of the quencher. The Stern-Volmer equation is formulated the following way (Stern and Volmer 1919):

\[
\frac{I_0}{I} - 1 = K_{SV} \cdot [Q]
\]

$I_0$: luminescence intensity of the indicator in the absence of the quencher; $I$: the luminescence intensity in the presence of the quencher; [Q]: the concentration of the quencher; KSV: Stern-Volmer constant

Figure 9 shows a plot of the Stern-Volmer relation and the change of the luminescence intensity of the optode against different oxygen saturations. Using this relationship the actual oxygen level in a sample can be determined.
The indicator and its polymer-matrix define the range of the measurement system. In most cases a metal-ligand complex is used for the indicator, because they have long decay times making the collisional quenching by O₂ more likely and sensing via measurement of decay times more simple (Wolfbeis 2015). However, the exact formulations are often not disclosed by the sensor manufacturers. Nevertheless, the most widely used fluorescent indicators for oxygen are:

- Platinum (II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PtTFPP)
- Palladium (II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PdTFPP)
- Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium (II) bis(perchlorate) (Ru(dpp)3

The indicators differ mainly in their decay times (τ) and luminosity (I) (Quaranta, Borisov, and Klimant 2012).

The polymer matrix is also very sensitive to the working principle of this measuring system. If the polymer exhibits high permeability for the dissolved oxygen molecules, the sensor can detect lower O₂ levels. The combination of an indicator with a long decay time with a highly permeable polymer leads to oxygen sensors that can be used to measure very low oxygen concentrations.
Optodes are capable of measuring both the intensity and the decay time. Since the measurement of the intensity is susceptible to interference, the oxygen calculations are generally based on the decay time ($\tau$). In general, the excitation of the indicator molecule is not carried out as a single signal but is performed sinusoidally (figure 10). The corresponding luminescence responses have also this sinusoidal character. The reduced signal intensity is linked to a phase shift ($\phi$). This phase shift is in a mathematical relationship with the Stern-Volmer calculation, so that the amount of oxygen can be calculated via the phase shift as well. The common user software in luminescence-based measuring systems corrects the oxygen amounts according to the temperature, the air pressure and possibly the salinity.

![Figure 10: Sigmoidal signals of oxygen measurements via optodes](image)

Different amounts of oxygen in the sample lead to changed sigmoidal curves. The deviations ($\phi$) from a reference value are referred to as phase deviation or the decay time.

### 3.1.7.2. SDR-Reader

According to the information provided by PreSens GmbH (consulted online on August 01, 2019 via www.presens.de), the SDR measuring system has the following properties: The SDR SensorDish® Reader from PreSens Precision Sensing GmbH is a small 24-channel reader for non-invasive detection of oxygen in multidishes (SensorDishes®) via luminescence quenching. The measurements can be performed manually or automated over long periods of time (days). The multidishes contain a sensor spot (indicator) at the bottom of each well. They are read out non-invasively through the transparent bottom. SensorDishes® for oxygen (OxoDish®) are available in 24-well and 6-well format. The SensorDish® Reader can be used directly in incubators. Up to 9 SDR Extension Sets can be combined with one Basic Set. The Extension Set
consists of a reader and a connection cable to install up to 10 devices in a row. Instead of having to change the plate on the reader monitoring can be carried out in up to 240 samples in parallel. All the SDR SensorDish® Reader can be controlled and read out with one software, that corrects the measured oxygen levels according to different temperatures, air pressures and salinity. There are also adaptations to dry measurements and measurements in wet media. A special feature of the SDR SensorDish® Reader is the possibility to determine pH values in parallel to the oxygen values. For this, the samples must be placed on HydroDish® plates. The multidishes are precalibrated, sterile and can perfectly be used for transmigration assays as performed in this work. The manufacturer gives the following data (table 4):

<table>
<thead>
<tr>
<th>Specifications</th>
<th>pH*</th>
<th>Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measuring range</td>
<td>6.0 - 8.5 pH</td>
<td>0 - 50 % O₂</td>
</tr>
<tr>
<td>Resolution*</td>
<td>± 0.05 pH at pH = 7</td>
<td>± 0.4 % O₂ at 20.9 % O₂</td>
</tr>
<tr>
<td>Precision*</td>
<td>± 0.2 pH at pH = 7 (sensor batch calibration)</td>
<td>± 1 % O₂ at 20.9 % O₂</td>
</tr>
<tr>
<td>Drift*</td>
<td>&lt; 0.1 pH within one week (sampling interval 10 min)</td>
<td>&lt; 0.2 % O₂ within one week (sampling interval 10 min)</td>
</tr>
<tr>
<td>Measurement temperature range</td>
<td>from +15°C to +45°C</td>
<td></td>
</tr>
<tr>
<td>Response time [t_{50}] at 25 °C</td>
<td>&lt; 30 sec.</td>
<td>&lt; 120 sec.</td>
</tr>
</tbody>
</table>

Table 4 Specifications of the SDR SensorDish® Reader

3.1.7.3. Oxy-1 ST

The Oxy-1 ST from PreSens Precision Sensing GmbH is a small and lightweight oxygen meter that can be used with microsensors (200 µm fiber) in different designs, non-invasive sensors, dipping probes and flow-through cells (1 mm fiber). OXY-1 ST is compatible with sensor type PST7 (detection limit 15 ppb, 0 - 100 % oxygen). It is operated with the PreSens Measurement
Studio 2 software and offers temperature, pressure and salinity compensated measurements as well as the SDR SensorDish® Reader. The software allows to control several OXY-1 ST simultaneously. Powered via USB no extra cables or adapters are needed. The Oxy-1 ST is connected directly to a computer via USB cable. The manufacturer gives the following data (table 5):

![Image](https://www.presens.de/products/detail/oxy-1-st.html)

Table 5 Specifications of the Oxy-1 ST

To determine oxygen levels in the cerebrospinal fluid, the measurement is performed with the Oxy-1 ST, which is connected via a polymer optical fiber (POF) to an O₂ Flow-Through Cell FTC-PST7 sensor. The manufacturer gives the following data for this sensor type (table 6):
3.1.7.4. **Parallel measurements of the pH with the pH-1 mini**

The pH-1 mini is a precise fiber optic pH meter that is optimized for measurements in physiological solutions. The measurement of the pH is also carried out by the principle of fluorescence quenching and is similar to the previously described principle for oxygen measurement. However, in acidic medium the fluorophore does not emit fluorescence, while in basic medium luminescence occurs. The pH sensor has a very short decay time that is difficult to measure. Therefore, a long-decaying, pH-independent reference dye is mixed in and the mixed signal is measured. This principle is called DLR (Dual Lifetime Referencing). By a sigmoid calibration curve the signal is converted to pH. The manufacturer gives the following data for this pH device (table 7):

![Table 6 Specifications of the O₂ Flow-Through Cell FTC-PST7 sensor](https://www.presens.de/products/detail/o2-flow-through-cell-ftc-pst7.html), accessed 29.07.2019
To determine pH in the cerebrospinal fluid, the measurement is performed with the ph-1 mini, that is connected via a polymer optical fiber (POF) to Single-Use pH Flow-Through Cell FTC-SU-HP5-US - sensor. The manufacturer gives the following data for this sensor type (table 8):

<table>
<thead>
<tr>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measurement range</strong></td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
</tr>
<tr>
<td><strong>Drift</strong></td>
</tr>
<tr>
<td><strong>Measurement temperature range</strong></td>
</tr>
<tr>
<td><strong>Response time (t50)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compatibility</strong></td>
</tr>
<tr>
<td><strong>Cross sensitivity</strong></td>
</tr>
<tr>
<td><strong>Cleaning procedure</strong></td>
</tr>
<tr>
<td><strong>Calibration</strong></td>
</tr>
<tr>
<td><strong>Storage stability</strong></td>
</tr>
</tbody>
</table>

Table 7 Specifications of the pH-1 mini

Table 8 Specifications of the pH Flow-Through Cell FTC-SU-HP5-US - sensor
3.1.8. The hypoxia chamber - generation of defined oxygen atmospheres

In order to change the oxygen content of the surrounding cell culture in a defined way, the so-called hypoxia chambers can be used. These chambers are similar to cell incubators, but they are hermetically sealed and may also meet special biosecurity requirements (for example the installation of HEPA filters). There are many different models on the market. In order to change the atmosphere, CO₂ and / or nitrogen can be introduced. The nitrogen displaces the oxygen, so that it can be adjusted. As in the normal cell incubator, the temperature and humidity can also be adjusted. In the present work, the “O₂ Control In Vitro Glove Box” (Coy Laboratory Products, Inc.) was used for special parts of the work (figure 1.11). The hypoxia chamber may allow to adjust cell culture models to defined oxygen conditions, especially hypoxic conditions as they may occur during infection and inflammation.

3.2. Streptococcus suis meningitis

The so-called Streptococcus (S.) suis meningitis is an infectious disease in pigs and humans, which can be triggered by the bacterium S. suis. To date, the pathomechanisms and the
processes of the immune system in a corresponding infection are only partially understood. A problem in the research on the pathogen-host interactions is a lack of suitable cell culture models to study the infection in vitro by mimicking the in vivo situation as best as possible.

3.2.1. Streptococcus suis

*Streptococcus (S.) suis* is a bacterium of the genus *Streptococcus* within the order Lactobacillales. It is a Gram-positive, facultative anaerobic bacterium. This means that *S. suis* usually grows under aerobic conditions but can also survive under anaerobic conditions due to a change in metabolism. It is catalase negative, immobile, forms pairs or chains and has a diameter of about 2 microns. In the fermentative metabolism lactate is mainly produced (Selbitz, Truyen, and Valentin-Weigand 2015).

The reservoir of *S. suis* are domestic pigs (Lowe et al. 2011) and wild boars (Baums et al. 2007; Verónica Sánchez del Rey et al. 2014). Nevertheless, the bacterium was also found in other animals species such as rabbits, dogs or lambs (Muckle et al. 2010, 2014; V. Sánchez del Rey et al. 2013).

The cultivation of *Streptococcus suis* can be performed in special liquid media, e.g. Todd Hewitt Broth (THB) (Todd and Hewitt 1932) or on blood agar plates at 37 °C, both aerobically and anaerobically. The bacterium shows white-greyish colonies on sheep blood agar with a diameter between 0.5 and 1 mm with an occurring α-hemolysis (Wewer 2009).

*S. suis* is a very heterogeneous bacterium with different serotypes and strains. There are currently 35 serotypes (ST) known that differ in their capsular antigenicity. However, for a few serotypes (ST 32 and ST 34), it has been suggested to assign them to a different bacterial species (Okura et al. 2016). In diseased pigs serotype 2 is the most prevalent worldwide followed by ST 9 and ST3 (Goyette-Desjardins et al. 2014).

Virulence factors help a pathogen to survive in the host organism (Cross 2008). *S. suis* is characterized by a variety of virulence factors (Fittipaldi et al. 2012), so it has e.g. a polysaccharide capsule to evade phagocytosis (Houde et al. 2012; M. Segura, Gottschalk, and Olivier 2004; Chabot-Roy et al. 2006). In addition it is discussed that the capsule helps the bacterium against entrapping by the so-called neutrophil extracellular traps (Zhao et al. 2015).
Further it was shown that *S. suis* is able to generate biofilms, which make them more resistant to certain antibiotics (Grenier, Grignon, and Gottschalk 2009). Another virulence factor is the exotoxin suilysin (SLY), which can destroy host cells by forming pores in their cell membranes (Barnett et al. 2015; Tenenbaum et al. 2016). This was - in addition to other cells - shown for porcine brain microvascular endothelial cells (pBMEC) (Vanier et al. 2004) or for human brain microvascular endothelial cells (hBMEC), in which the mechanism of an alteration of the host’s actin cytoskeleton due to the influence of SLY was discovered (Lv et al. 2014).

The mechanisms of how *S. suis* exactly adapts in the body of the host are largely unknown (Jörg Willenborg and Goethe 2016). However, transcriptome analyses of *S. suis* under infectious conditions have been recently studied *in vitro* and demonstrated differences in the activities of the amino acid metabolism for example between the medium blood and the medium cerebrospinal fluid (Koczula et al. 2017).

*S. suis* is a commensal bacterium predominantly in the upper respiratory tract (tonsils and nasal cavities), but also in the genital and digestive tracts (Robertson and Blackmore 1989; Gottschalk and Segura 2000). However, synergistic interaction with other agents may increase the risk of invasive infection (Meng et al. 2015) that can cause severe symptoms such as septicaemia, arthritis, endocarditis, pneumonia or meningitis or even a per acute dying (Sanford and Tilker 1982; Gottschalk et al. 2010; Arends et al. 1984).

Compared with the high spread of the germ in pig herds (prevalence about 100 %), severe symptoms in pigs are rarely observed (Goyette-Desjardins et al. 2014). Infected but healthy animals are of great relevance as they function as transmitters. Horizontal transmission is usually via nose-to-nose contact (Dekker et al. 2013), but vertical transmission between the sow and the piglet is also possible during phase of farrowing (Amass, SanMiguel, and Clark 1997). Clinical symptoms of infection are most prevalent in pigs at weaning age.

The bacterium shows also a zoonotic character: A *S. suis* infection in a human was first described in Denmark in 1968 (Arends and Zanen 1988). In contrast to pigs *S. suis* in humans usually causes most bacterial meningitis, nevertheless, other symptoms such as septicaemia, pneumonia, endocarditis or peritonitis may also occur (Thi Hoang Mai et al. 2008; Suankratay et al. 2004; Gottschalk et al. 2010). In addition, a late episode of the infection can be deafness (Thi Hoang Mai et al. 2008). *S. suis* ST 2 is most commonly found in human *S. suis* infections with 97% (Goyette-Desjardins et al. 2014). A major outbreak of the disease in China in the year
2005 resulted in a mortality rate of approximately 20% (Gottschalk, Segura, and Xu 2007). However, compared to pigs, the prevalence of S. suis in the human population is estimated to be only about 5% and carriers are believed to be in intensive contact with pigs (e.g., farmers, butchers, veterinarians) (Strangmann, Fröleke, and Kohse 2002; Goyette-Desjardins et al. 2014), although cases of human infection without previous contact to pigs have been reported (Kerdsin et al. 2016).

People can be infected by the distortion of raw pork (Fongcom et al. 2001) or by small skin wounds that allow the bacteria to enter the bloodstream (Wertheim et al. 2009). The disruption of epithelial barriers is considered to be the first step of invasion of the bacteria into the bloodstream (Mariela Segura et al. 2016). After having successfully entered the blood the bacteria spread via the blood flow in the whole body and can reach the brain structures or the cerebrospinal fluid (CSF) after breaking through the brain barriers (K. S. Kim 2008; Pulzova, Bhide, and Andrej 2009) and thus initiate the early phase of meningitis (Gottschalk and Segura 2000; Fittipaldi et al. 2012; Mariela Segura et al. 2016). Several studies have shown that especially the choroid plexus epithelium of the blood-cerebrospinal fluid barrier (BCSFB) is used as an entry into the CSF compartment of S. suis (Schwerk et al. 2015; Wewer et al. 2011; Schwerk et al. 2012).

To overcome the brain barriers there are three possibilities most mentioned for the bacteria: transcellularly, paracellularly or attached to phagocytotic cells, which transmigrate through the barriers (so-called "Trojan Horse Theory") (K. S. Kim 2008; Pulzova, Bhide, and Andrej 2009; Dando et al. 2014). This theory has been broadened to postulate that S. suis attaches to non-disease-associated phagocytes that transmigrate normally with a small number into the CSF. By this mechanism they reach the CSF compartment where they start to replicate. This is called the "modified Trojan horse theory" (Gottschalk and Segura 2000).

At present it is still unclear why a disease leads to meningitis in some individuals, but leads to different symptoms or no disease in others (Higgins et al. 1990). In addition to the further investigation of the pathogen itself, intensified research on the pathogen-host interaction, regarding the immune system, should be underlined here.

In the following work S. suis Serotype 2, strain 10 is used for infection studies in vivo and in vitro. This strain was cultivated and published in 1999 at the DLO Institute for Animal Science.
and Health, Lelystad, The Netherlands (Smith et al. 1999). It was shown that *S. suis* Serotype 2, strain 10 is highly virulent in experimental infections of piglets (Vecht et al. 1989). Therefore, it was used by many research groups in experimental infections *in vivo* and *in vitro* (J. Seele et al. 2013; de Buhr et al. 2016, 2015; Seitz et al. 2012; Jana Seele et al. 2015).

### 3.2.2. Meningitis

The brain and the spinal cord are surrounded by several meninges (figure 1.12). These are from the inside out the so-called *pia mater*, the *arachnoid mater* and the *dura mater*. While the pia mater is firmly connected to the brain tissue with its cerebral furrows, the *arachnoid* is only loosely connected to the *pia mater* and forms with this a space, the so-called *subarachnoid space*. The outer layer, the *dura mater*, rests firmly on the *arachnoid* and is also firmly connected to the cranial bone that is followed by the periosteum, the aponeurosis and finally the skin. The subarachnoid space is filled with the cerebrospinal fluid (CSF) and is called the “outer cerebrospinal fluid space”.

An inflammation of these tissue layers is called “meningitis”. It can be infectious and caused by special bacteria, viruses or fungi, as well as having a non-infectious cause (Nau et al. 2014; Ginsberg 2004) (e.g. autoimmune diseases or cancer).

Due to their proximity to the brain, bacterial meningitis in particular is dangerous and can lead to death if left untreated (Tunkel et al. 2004). Nonetheless, long-term damage such as deafness, epilepsy or cognitive impairment can occur (Sáez-Llorens and McCracken 2003; van de Beek et al. 2006).

Besides S. suis (as mentioned above) also numerous other bacteria can lead to meningitis, often in an age-dependent manner. The common meningitis-causing pathogens are: *Streptococcus agalactiae*, *Escherichia coli*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Neisseria meningitidis* (Huang, Brady, and Mortensen 2019).

Normally, the infectious agents reach the meninges via the bloodstream (alternatively, direct infections, e.g. as a result of an injury or iatrogenic reasons can also occur). To overcome the brain barriers, the pathogens must be present in a reasonably high amount (K. S. Kim 2003).

The bacterial meningitis is normally characterized by a high influx of neutrophils into the tissues, hyperaemic blood vessels and partially combined with an inflammation of the choroid plexus (choroiditis) (Y. Reams et al. 1994). However, how pathogens penetrate the blood-brain barrier (BBB) or the blood-cerebrospinal fluid barrier (BCSFB) is poorly understood so far as well as the following mechanisms of the host-pathogen immune interactions.

### 3.2.3. The blood-cerebrospinal fluid barrier (BCSFB) and the choroid plexus

The nervous system is one of the partially shielded organs from the immune system, an immune status called “immune privilege” (Louveau, Harris, and Kipnis 2015). A major reason for the immune privilege of the nervous system are the barriers that separates the blood from the parenchyma. They are formed by closely linked endothelial or epithelial cells. They prevent the uncontrolled entry of circulating immune cells and serum proteins such as immunoglobulins. They also protect the brain and spinal cord from harmful substances such as pathogens or toxins. Nevertheless, in some diseased cases this protective function is not effective and the barrier maybe become leaky.

The most well-known barrier is the “real” blood-brain barrier (BBB), which separates the blood vessels from the brain parenchyma. Less known is the so-called blood-leptomeningeal barrier (BLMB). This barrier is formed by tightly connected blood vessels in the subarachnoid space, thus a barrier between the pia mater and the arachnoid, collectively referred to as
“leptomeninx”. The blood vessels in these two barriers are formed by a tight junctions connected endothelium (Welsch 2014). This is in contrast to the third barrier, the so-called blood-cerebrospinal fluid barrier (BCSFB):

The BCSFB is formed by an endothelium, loose connective tissue and a tight connected epithelium. Due to the fenestration of the BCSFB-endothelium it does not contribute to the barrier function, instead this is done by the choroid plexus epithelial cells (Wolburg and Paulus 2010). In addition, this epithelium is the main producer of the cerebrospinal fluid (CSF). The polarized epithelium of the BCSFB together with the blood vessels and their surrounding connective tissue is collectively referred as choroid plexus (CP) (figure 1.13). Since the choroid plexus epithelial cells represent the morphological barrier correlate to the BBB’s tight endothelial cells, in vitro models of the BCSFB are based on these cells (Schwerk et al. 2015). The choroid plexus epithelial cells increase their surface by apical microvilli towards the CSF compartment (Rediz and Segal 2004) and have numerous mitochondria to meet their high energy requirements due to further secretion and transport functions for the CSF production (Cornford et al. 1997).

The CSF producing CP can be found in all the four ventricles inside the brain (two paired lateral ventricles, the unpaired third ventricle and the fourth ventricle near the cerebellum). These
four brain ventricles communicate with each other through diverse connections (Welsch 2014) like the two *foramina interventriculares* that link the lateral ventricles with the third one, the *aquaeductus mesencephalic* (cerebral aqueduct) connecting the third and fourth ventricle. By the two foramina of *Luschka* (lateral apertures) a channel for CSF to flow from the brain’s ventricular system into the subarachnoid space is provided. Through the foramen of *Magendie* (median aperture) cerebrospinal fluid flows into the *cisterna cerebellomedullaris* (figure 1.14), a suitable structure for obtaining CSF samples by puncture (D’Angelo et al. 2009). Finally, the CSF flows surrounding the spinal cord (and inside the central canal) towards the pelvis.

![Figure 14: CSF circulation inside the CNS](https://commons.wikimedia.org/wiki/File:1317_CFS_Circulation.jpg)

The four choroid plexus inside the ventricles produce the cerebrospinal fluid that circulates between different structures in the brain and spinal cord and is finally venously resorbed in the *granulationes arachnoideales*. Graph adapted from https://commons.wikimedia.org/wiki/File:1317_CFS_Circulation.jpg (licensed under CC-BY-4.0, accessed 10.08.2019)

The blood-brain barrier and the blood-cerebrospinal fluid barrier differ in their quality and permeability to certain substances. For example, the BCSFB has been shown to be restrictive to horseradish peroxidase (often used for biotechnological analysis), but unlike the BBB, it allows for the paracellular passage of lanthanum, a drug (lanthanum carbonate) for lowering the phosphate level in patients (Tonelli, Pannu, and Manns 2010). Furthermore, it has been shown that an agent used in HIV therapy entered the brain via the BCSFB, but the BBB itself is
impassable for the substance (stavudine) (Thomas and Segal 1998). Such observations illustrate the possibly therapeutic potential of the BCSFB to carry drugs into the brain under bypassing the BBB (Dragunow 2013). Nevertheless, some pathogens also use the BCSFB as an entry portal into the CNS. For viruses, these are e.g. coxsackie virus B3 (CVB3) (Feuer et al. 2003), lymphocytic choriomeningitis virus (LCMV) (Puccini et al. 2013), human immunodeficiency virus (HIV) (Falangola et al. 1995) or the simian immunodeficiency virus (SIV) (Lackner et al. 1991). The invasion by bacteria was for example shown for *Haemophilus influenzae* (Daum et al. 1978), *Streptococcus suis* (Sanford 1987) and *Neisseria meningitidis* (Pron et al. 1997; Schwerk et al. 2012). Also for parasites, such as *Trypanosoma brucei*, a CP involvement was shown (Masocha and Kristensson 2012).

### 3.2.4. The cerebrospinal fluid (CSF)

The cerebrospinal fluid is produced by the CP epithelium via ultrafiltration of the blood and active transport mechanisms especially of sodium ions (Damkier, Brown, and Praetorius 2013; Wolburg and Paulus 2010) and is mostly resorbed by root pockets of the cerebral and spinal cord nerves as well as the *granulationes arachnoideales*, that are protuberances from the outer CSF compartment into the dural venous sinuses (Johanson et al. 2008) (figure 1.12). The volume of CSF of an adult human is about 150 ml on average. Since approximately 500-600 ml are formed during 24 hours, this means that the CSF in humans is exchanged about 3-4 times a day (Wright 1978). The CSF formation rate in humans vary from 0.3 to 0.6 ml/min (Johanson et al. 2008), for pigs it is estimated at 0.9 ml/min but differences upon the breed and the size of the pigs must be taken into account (Stromholm et al. 1994). The pressure of the CSF in humans (lying position) is about 5-15 mmHg (Zettl 2005) in pigs the CSF pressure is stated to about 8 mmHg (Kaiser and Frühauf 2007).

In humans, several methods for CSF collection are described (Zettl 2005): The most common method is the lumbar puncture (sitting or lying position), in which up to 15 ml of CSF can be taken from the adult per procedure. Also possible are the medial or lateral suboccipital (atlantooccipital) puncture of the *cisterna cerebellomedullaris* but both should be performed under fluoroscopy. As part of surgical intervention, direct ventricular puncture through the bone is also possible.
In pigs the lumbar puncture and the atlantooccipital puncture of the *cisterna cerebellomedullaris* are possible methods to collect CSF. For both collection methods the animals must undergo anaesthesia.

Physiological cerebrospinal fluid is clear and transparent. At high cell counts (> 500 / µl) turbidity and an increase in viscosity can occur. It contains very little protein, which mainly consists of albumin (coming from the liver) (Moritz 2013). The quantitative determination of proteins in cerebrospinal fluid without reference to the blood values is not very informative, therefore both values should be considered in correlation. Under normal conditions, the protein content in serum is about 200 times higher than in the cerebrospinal fluid. The CSF-serum quotient for human albumin is up to $8 \times 10^3$. Higher values point to a disturbance of the BCSFB (Zettl 2005). Table 9 shows some parameter in the CSF of humans.

<table>
<thead>
<tr>
<th>parameter</th>
<th>blood</th>
<th>CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>$7.394 \pm 0.017$</td>
<td>$7.306 \pm 0.028$</td>
</tr>
<tr>
<td>glucose [mmol/l]</td>
<td>$3.9 – 5.5$</td>
<td>$2.7 – 4.2$</td>
</tr>
<tr>
<td>lactate [mmol/l]</td>
<td>$0.5 – 2.2$</td>
<td>$1.2 – 2.1$</td>
</tr>
<tr>
<td>total protein [g/l]</td>
<td>$70$</td>
<td>$0.2 - 0.5$</td>
</tr>
<tr>
<td>albumin [g/l]</td>
<td>$35 - 55$</td>
<td>$0.15 – 0.35$</td>
</tr>
</tbody>
</table>

**Table 9** *Selected parameter in the CSF of healthy humans (Zettl et al. 2005)*

In addition to its protective mechanical function the cerebrospinal fluid distributes numerous hormones, growth factors and transmitters in the brain, providing the basis for “CNS internal communication” (Adelman and Smith 1999; N Lai et al. 1991; Zemo and McCabe 2001) and is in addition a medium for transporting iron in and out of the brain (Lu, Kaur, and Ling 1995; Moos and Morgan 2000). One prominent example for the hormone secretion by CP into the CSF is the vascular endothelial growth factor (VEGF) (Sivakumar et al. 2008; Yang et al. 2010), a hormone that stimulates the angiogenesis in case of hypoxia (Shweiki et al. 1992). Further it was demonstrated in a rat model that neutrophils migrate into the brain parenchyma by the way of the CP after the cells expressed certain chemokines which influenced the infiltration of the neutrophils, e.g. the cytokine-induced neutrophil chemotactant (CINC)-1 (Szmydynger-
3.2.5. Neutrophil granulocytes

The innate immune system consists of different immune cells. One group of cells are the so-called “granulocytes” that are separated into neutrophilic, eosinophilic or basophilic granulocytes. These cells are characterized by a distinct presence of granules (vesicles with different mediators). Neutrophil granulocytes make up the majority of white blood cells circulating in the blood (50-70%). The cell nuclei of the neutrophils vary in their shape. In the early stages of maturation, the cell nuclei are lobed, while in the differentiated stage they have segmental structures. This property led to the further designation of polymorphnuclear (PMN) granulocytes (Alberts et al. 2017).

The neutrophils, which occur only for a short time in the blood with a half-life span of about 6-10 hours (McCracken and Allen 2014; Summers et al. 2010) or at least less than 24 hours (Lahoz-Beneytez et al. 2016) are formed in the bone marrow. If there is a need for their appearance in case of infection an increased release of immature neutrophils can be observed (Kolaczkowska and Kubes 2013). They can be quickly mobilized into the tissue. After mobilization into the tissue, they can accumulate in large amounts. The hypoxia, which often occurs locally in infectious areas, can be managed by the neutrophil granulocytes by intensive anaerobic glycolysis. Therefore they have large storages of glycogen (Horn 2012).

If there is no recruitment due to an infection, the cells will die after 1-2 days (Martin and Resch 2009). Because of their short lifespan, neutrophil granulocytes are constantly produced.

PMN belong to the “first line of defense” (Chin and Parkos 2007), fight against pathogens via different mechanisms and also play an important regulatory role (Mantovani et al. 2011). In order to achieve infected tissue PMN leave the bloodstream and its vessels in a multi-stage process mediated by adhesion molecules and chemokines. According to today's knowledge, the processes are shortened as follows (Baumgärtner and Gruber 2015; Muller 2013):

After tissue macrophages or other cells have sensed pathogens, they release cytokines such as Interleukin (IL)-1 and Tumor Necrosis Factor (TNF) α. This causes the expression of certain molecules and selectins in nearby endothelial cells mainly of postcapillary venules parallelly to an altered blood flow velocity with corresponding repositioning of the PMN inside the
vessels (margination). A stimulated expression of E-selectin (1-2 hours) and P-selectin (very fast) by the endothelial cells leads to a linkage with special sialinated oligosaccharides of the PMN (rolling). As further ligands, Intercellular Adhaesion Molecule-1 (ICAM-1) and Vascular cell adhesion molecule-1 (VCAM-1) are expressed, which - in particular - bind to macrophages and lymphocytes. The Platelet endothelial cell adhesion molecule-1 (PECAM-1) as well as Junctional Adhaesion Molecules (JAM) are further important adhesion molecules in the following process of transmigration, named diapedesis. The diapedesis is an important factor in the activation of PMN and leads to altered expression of antigens on the surface (Murphy 2016). The PMN migrate along a concentration gradient of chemokines through the tissue to the site of infection. N-formyl-methionine is for example a bacterial chemoattractant that stimulates PMN, examples for an endogenous substance with a stimulating effect are the CSa component of the complement system, leukotrienes (LTB₄) and chemokines, such as IL-8.

However, the adhesion molecules that play a role in the migration of PMN via endothelia in the CNS or via epithelial cells (e.g. trough the BCFB formed by choroid plexus epithelial cells) are only speculative so far (Kolaczkowska and Kubes 2013).

Neutrophil granulocytes fight pathogens in various ways. First, the so-called phagocytosis phenomenon, also occurring in PMN, was discovered by the Russian scientist Élie Metchnikoff (1845–1916) in the year 1880 (Rosales and Uribe-Querol 2017). Here, pathogens are integrated into so-called phagosomes, which fuse in the cell with lysosomes and become the so-called phagolysosome. Here the pathogens are digested lysosomally by various degrading enzymes (Rosales and Uribe-Querol 2017). The PMN recognizes special conserved pathogen associated molecular patterns (PAMPS) via pattern recognition receptors (PRR). These PAMPS are often components of the bacterial cell membranes. Another mechanism is the detection of pathogens that were previously opsonized by other immune cells or the complement system. These opsonizations are registered by PMN by the so-called fragmented crystallizable (Fc)- receptors (Murphy 2016).

The second way, PMN fight against pathogens is the phenomenon of degranulation. Hereby the PMN release granules with antimicrobial substances and proteases (Lacy 2006; Lominadze et al. 2005) that can degrade virulence factors or directly kill the pathogens in the tissue.

Another recently discovered third mechanism is the release of neutrophil extracellular traps (NETs) (Brinkmann 2004). The major component of the NETs is chromosomal DNA, which is
decorated with antimicrobial peptides and enzymes such as myeloperoxidase (MPO), neutrophil elastase, cathepsin G, lactoferrin and gelatinase. In addition, different histones form the backbone of the NETs (Brinkmann 2004). Pathogens are trapped by the network structure of the NETs, in where they are exposed to a high concentration of the antimicrobial molecules. It was shown that PMN release this kind of traps in case of a S. suis infection – also in the cerebrospinal fluid (de Buhr et al. 2016). A bacterial defense mechanism against these DNA structures is to destroy them via special DNases and thus escaping the entrapping. In studies on the host-pathogen interaction, this could be shown also for S. suis (de Buhr et al. 2015).

The physiological amounts of neutrophil granulocytes in the blood and cerebrospinal fluid in humans and pigs are shown in tables 10 and 11.

<table>
<thead>
<tr>
<th>species</th>
<th>lobed neutrophils</th>
<th>segmented neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relative [%]</td>
<td>neutrophils/µl</td>
</tr>
<tr>
<td>human¹</td>
<td>3-5 (adults)</td>
<td>150-400 (adults)</td>
</tr>
<tr>
<td></td>
<td>0-10 (children)</td>
<td>0-1200 (children)</td>
</tr>
<tr>
<td>pig²</td>
<td>0-7</td>
<td>0-1500</td>
</tr>
</tbody>
</table>

Table 10 Neutrophil granulocytes in the blood in humans and pigs
¹ source: http://www.laborlexikon.de/Lexikon/Infoframe/b/Blutbild_gross.htm, accessed: 12.08.2019
² source: Klinische Labordiagnostik in der Tiermedizin, 7.Auflage, Schattauer

<table>
<thead>
<tr>
<th>species</th>
<th>cells/µl (total)</th>
<th>PMN [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>human¹</td>
<td>&lt;5 (lumbar)</td>
<td>0 (lumbar)</td>
</tr>
<tr>
<td></td>
<td>&lt;3 (suboccipital)</td>
<td>0 (suboccipital)</td>
</tr>
<tr>
<td>pigs²</td>
<td>&lt;14</td>
<td>0-5</td>
</tr>
</tbody>
</table>

Table 11 Cells and PMN in the CSF of healthy humans and pigs
¹ Source: Klinische Liquordiagnostik, 2. Auflage, de Gruyter
² Source: Schweinekrankheiten, Ulmer UTB

3.3. A cell culture model to study the BCSFB

To invade and subsequently trigger inflammation in the brain, the pathogens must overcome brain barriers such as the blood-brain barrier (BBB) or the blood-cerebrospinal fluid barrier (BCSFB). While there were already longer existing human models with immortalized cells for
the BBB available (Stins, Badger, and Sik Kim 2001; Weksler et al. 2005; Muruganandam et al. 1997), a cell culture model for mimicking the blood-cerebrospinal fluid barrier was developed later. First, there were already in vitro models using rat cells (Zheng and Zhao 2002) and primary porcine choroid plexus epithelial cells (PCPEC) available (Haselbach et al. 2001). However, a human choroid plexus cell model, which had its origin in a human choroid plexus carcinoma (KUMABE et al. 1996; Takahashi et al. 1997), did not form continuous tight junctions (Szmydynger-Chodobska et al. 2007), that are an elementary factor in the functionality of BCSFB.

A commercially available primary choroid plexus epithelial cell (HCPEpiC) line is offered by the company ScienCell Research Laboratories (Carlsbad, USA). This model has been used for studies on interactions between the CP, the CSF and the CNS in total (Gonzalez et al. 2011) and for the investigation of certain secretions (Pagliaccetti and Robek 2010). The use of these cells as a basis for infection studies with pathogens is not known.

Based on cells of a human choroid plexus papilloma cell line (HIBCPP) that were taken in the year 1995 from a patient in Japan who had this very rare tumor (Ishiwata et al. 2005), a cell culture model was published which did not show the above described deficiencies (Schwerk et al. 2012). This cell culture system is built on an 'inverted' Transwell filter system which is based on a porcine choroid plexus epithelial cell model (PCPEC) (Tenenbaum et al. 2009). It could be shown that the HIBCPP model forms tight junctions that are associated with a high transepithelial electrical resistance (TEER) and a low flow rate of macromolecules such as Inulin or Dextran; in addition, the barrier properties depend on different supplementation amounts of fetal calve serum (FCS). Well-formed tight junctions could be detected by immunofluorescence microscopy at TEER values around 500 $\Omega \times \text{cm}^2$, values that are comparable to in vivo investigations (Saito and Wright 1983; Villalobos, Miller, and Renfro 2002). In addition, expression of typical transcripts was detected by RT-PCR, which encode junctional proteins, like Claudin 1-3, Zonula occludens-1, Occludin and E-Cadherin (Schwerk et al. 2012). The cells form a monolayer in most areas on the membrane of the transwell filter, but nevertheless, occasionally they show growth in papillary-like structures. In addition, the nuclei show a pleomorphic morphology. These are phenomenons that are also described in the initial characterization of this cell line (Ishiwata et al. 2005).
It was shown, that *S. suis* transmigrate through the HIBC Plant barrier significantly stronger when the cells are applied in the inverted system than compared to non-inverted ("standard") system (Schwerk et al. 2012). Incubation of the HIBC Plant on an inverted transwell filter system allows experimental infections of cells from the physiological side - basolateral side. The microvilli hem formed by the cells points to the experimental "CSF compartment", the basolateral side is aligned to the "blood compartment" (figure 1.15).

![Diagram of HIBC Plant on an inverted transwell filter system](image)

**Figure 15** HIBC Plant on an inverted transwell filter system
This arrangement allows experimental infections from the physiological (basolateral) side. The cells form a barrier between the upper "blood compartment" and the lower "CSF compartment"

### 3.4. Pigs as in vivo models in neuroscience

Rodents and primates have been the preferred animals in research in the field of neuroscience for a long time. In the field of toxicology research however (Lehmann 1998; Svendsen 2006; Bode et al. 2010) and the development of new surgical techniques (Richer et al. 1998), the number of pigs was increasing significantly over the years. That can be explained by the expanding pig production and therefore the good and fast availability, as also by the similarities between porcine and human anatomy and physiology (Bustad and McClellan 1965; Douglas 1972). For the study of brain diseases in early childhood, the pig was discovered to be a suitable animal model (Glauser 1966). In their work Lind et al. list numerous areas of neurological research in which pigs serve as experimental animals today. Comparisons as well as differences to humans have been highlighted (Lind et al. 2007). For example, the porcine model was used to study the influence of malnutrition (Ng and Innis 2003) or the chronic consumption of alcohol on the human brain (Harris 1983). In addition, there have been promising studies on the possible use of porcine brain tissue in degenerative diseases of the
human brain (Deacon et al. 1997). Special porcine models for the study of Parkinson's disease (Mikkelsen et al. 1999) or Alzheimer's disease (Lee et al. 2017) have been developed. In addition, there are numerous porcine models for internal and surgical intervention in traumatic brain injury in humans (Armstead and Kurth 1994; Duhaime et al. 2003; Durham et al. 2000; Munkeby et al. 2004).
The pigs used in the research usually come from the Eurasian wild boar (*Sus scrofa*). There are numerous national and regional varieties that are used primarily in food production, such as Yorkshire, Hampshire or Duroc (Michael Swindle and Smith 2008). In addition, in Germany, the German Landrace (GL) and the German Large White (LW) or more often hybrids from the latter are bred for the meat market. Hybrids have the advantage that they often combine several positive features for the market, such as health, reproduction rates or rapid weight gain.
The disadvantage of the use of such "agricultural pigs" in biomedical research, however, lies precisely in the rapid weight gain, so that these animals are often used only for short-term experiments. In addition, poor standardization of the different breeding lines can be a problem. Therefore, special - in part standardized - breeds for use in research have been put on the market, e.g. the Göttingen Minipig (McAnulty et al. 2011). Meanwhile, there are also numerous genetically modified pigs for the investigation of certain diseases available (Perleberg, Kind, and Schnieke 2018).

3.5. Aims of the study

The aim of this work is to determine the oxygen level occurring in case of a *S. suis* meningitis in the cerebrospinal fluid of pigs *in vivo* and to compare these data with the actual occurring oxygen level, that were measured *in vitro* on a cell culture model, that mimics the blood-cerebrospinal fluid barrier. The detection of possible deviations in the oxygen level between the *in vivo* situation and the *in vitro* situation should help to adapt the cell culture system better to the real-life conditions.

A later adaptation of the oxygen levels in the cell culture can help to carry out infection studies on a further optimized model and thus to achieve more transferable results.
The goal of this work is to further reduce the use of previously necessary animal experiments by optimizing cell culture as an alternative method to researching the host-pathogen interaction in the early phase of meningitis.
4. Methods

4.1. *In vitro*: Studies on the cell culture

4.1.1. Preparation of *S. suis* stocks

*S. suis* serotype 2 strain 10 (Smith et al. 1999) was smeared on a Columbia agar plate with 6% sheep blood (Oxoid Deutschland GmbH) and was incubated for 16 – 18 hours at 37°C. After this time a single bacterial colony was picked and added into 10 ml Todd Hewitt Broth (THB) (Becton, Dickinson and Company, Sparks Glencoe, USA) in a T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. The suspension was ice-stored incubated for 17 hours, leading to a delayed start of growth. Afterwards a 1:50 dilution was created, and the bacteria grew until reaching the mid log phase (OD 0.6) determined by photometric measurements at 600 nm (JENWAY 6310; Jenway, Stone, UK). The suspension was supplemented with glycerol to a final concentration of 15%. The mixture was aliquoted in 500 µl portions and further bacterial growth was immediately stopped by shock freezing in liquid nitrogen and long-time stored at -80°C. The quality of the stocks was checked by performing representative fragmented smears of melted cryostocks on blood agar plates to control the bacterial vitality and to check for contamination.

Three independent bacterial cryostocks (batches 1-3) were created by this method at different times. Afterwards, determinations of the colony forming units (CFU/ml) were performed to quantify the bacterial number of each batch (chapter 4.1.2).

4.1.2. Determination of colony forming units (CFU)

The colony forming units (CFU) per ml of bacterial samples were determined by plating on agar plates in different dilutions. To create a dilution series, 20 µl sample was added to 180 µl phosphate buffered saline (PBS). From this first 1:10 dilution (10⁻¹) 20 µl were added again to 180 µl PBS to dilute to 1:100 (10⁻²). The dilution series was continued according to this principle until a defined dilution. Thorough homogenization between the steps is achieved by pipetting up and down at least 7 times. It is necessary to change the pipette tip between different dilutions.
With a pipette 20 µl of the different dilutions were added to one-hour preheated Columbia agar plates with 6% sheep blood (Oxoid Deutschland GmbH) if not stated otherwise and are distributed in a line while rotating the plate until the drops were drawn into the agar. The applications on the plates were each done as a duplicate. Plating must be performed within 30 minutes.

Afterwards, the blood agar plates were incubated at 37 °C in the incubator for at least 16 hours (if not specified otherwise) until the bacterial colonies are visible, well countable and well differentiable according to their morphology if needed. Subsequently, the duplicated smears were examined, and the bacterial specific colonies were counted and averaged per plate. Including the dilution factor and the applied volume per smear the colony forming units (CFU) were calculated per 1 ml. For the calculation at least two different dilution plates were quantified with 5 – 200 colonies each.

4.1.3. Isolation of human neutrophils

In agreement with the local ethical board (permit no. 3295-2016, Hannover Medical School) blood from healthy donors was aspirated into lithium-heparin tubes (S-Monovette® 9ml LH, SARSTEDT AG & Co. KG, Nümbrecht, Germany). By the following procedure neutrophilic granulocytes (PMN) were separated by density gradient centrifugation:

In a 50 ml tube (SARSTEDT AG & Co. KG, Nümbrecht, Germany) a volume of 20 ml blood is put on top of 20 ml Polymorphprep™ (Alere Technologies AS, Oslo, Norway) without mixing. The tube is centrifuged for 30 minutes at 470 g with deceleration modus. Afterwards the mononuclear cells and the yellowish plasma layer are removed carefully with a pipette. The polymorphonuclear cell (PMN) phase of the density gradient is transferred carefully into a new 50 ml tube. The tube is supplemented with phosphate-buffered saline (Sigma-Aldrich, 10x concentrate, diluted 1:10 in endotoxin-free water, Carl Roth) up to 50 ml. The tube is centrifuged again for 10 minutes at 470 g with brake modus. The supernatant is removed, and the pellet is resuspended in 5 ml of endotoxin-free water for 5 seconds to lyse the erythrocytes. After the lysis a fill-up to 50 ml is immediately performed with PBS. It is followed by another centrifugation for 10 minutes at 470 g with brake modus. The supernatant is removed, and the pellet should be white without
erythrocytes. If there is still a red color (erythrocytes) the lysis step was repeated once. The white pellet is resuspended in 1 ml of Roswell Park Memorial Institute (RPMI) medium 1640 (without phenolred, PAA Laboratories, Inc., Cambridge, UK) and the concentration of PMN was determined. Therefore 10 μl suspension was supplemented with 90 μl 0.4% trypan blue (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to create a 1:10 dilution. From this, 10 μl were added to a haemocytometer (Neubauer counting chamber) and the number of PMN was determined according to the following formula 2.1:

\[
\text{Eq. 2.1} \quad \frac{\text{cells}}{\text{ml}} = \left(\frac{\text{cells in large squares}}{(\text{number of large counted squares})} \times \text{(dilution factor)}\right) \times 10000
\]

4.1.4. Preparation of the HIBCPP cell culture and conditions

The cell culture medium that was used for the HIBCPP incubation (37°C, 5% CO₂ and increased humidity due to an installed water bowl) was used in two variants - one with antibiotics and one without antibiotics. For all HIBCPP incubations that took place before the S. suis infections, the cell culture medium was composed as follows:

**15% FCS – cell culture medium:**

500 ml Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 1:1 with phenol red (DMEM/F-12; ThermoFisher, Waltham, USA) was used as basis, supplemented with 90 ml heat-inactivated fetal calf serum (FBS Superior S 0615; Biochrom GmbH, Berlin, Germany). The inactivation was performed by heating at 56°C for 30 minutes in a water bath. In addition, 12 ml of 200 mM L-glutamine (ThermoFisher, Waltham, USA) and 250 μl insulin (concentration 10 mg/ml) (Sigma-Aldrich Corp., St. Louis, USA) was added. This mixture was stored at +7°C in the refrigerator for maximal 1 month.

Weekly portions of 50 ml were aliquoted and were supplemented with 500 μl of penicillin/streptomycin (100x-concentrate; 10000 μg/ml; Biochrom GmbH, Berlin, Germany). The shelf life was a maximum of 1 week.

The cell culture medium that was used one day before and during the infection experiments was prepared as follows:
1% FCS – cell culture medium (free of antibiotics):

500 ml Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 1:1 without phenol red (DMEM/F-12; ThermoFisher, Waltham, USA) was used as basis, supplemented with 5 ml heat-inactivated fetal calf serum (FBS Superior S 0615; Biochrom GmbH, Berlin, Germany) and 250 μl insulin (concentration 10 mg/ml) (Sigma-Aldrich Corp. St. Louis, USA).

The human choroid plexus papilloma cells (HIBCPP) (Ishiwata et al. 2005; Schwerk et al. 2012) were stored as a cryostock in -150°C. To use the HIBCPP for the cell culture model, the cryostocks were first defrosted in a water bath at 37 °C until the ice started to melt. It was then immediately transferred to a tissue culture flask T-75 (SARSTEDT AG & Co. KG, Nümbrecht, Germany) filled with 15 ml of 37 °C prewarmed cell culture medium (15% FCS-medium). The cells grew with regular change of the medium – three times per week - for about 3 weeks until reaching about 90% of confluency. During that time the cells were examined regularly macroscopically and microscopically examined for any hints of contamination, such as turbidity, color changes of the cell medium or odor. Mycoplasma contamination was also regularly excluded by PCR analysis during routine processes (Mycoplasma Detection Kit; SouthernBiotech, Birmingham, USA). The cells were harvested at time of about 90% of confluency to maintain the cell line. Detachment was carried out according to the following principle:

The cell culture medium was carefully aspirated with a sterile Pasteur pipette. The cells were washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS; ThermoFisher, Waltham, USA). Subsequently, 3 ml of 0.25% trypsin-EDTA (ThermoFisher, Waltham, USA) was added to the cells for 15-20 minutes. Separation of the cells was sometimes assisted by punching carefully the cell culture flask. After the cells became detached, 7 ml of cell culture medium was added and mixed thoroughly with the cells by pipetting up and down. The suspension was then centrifuged at 250g for 10 minutes at room temperature without acceleration or deceleration modus. The supernatant was removed with a sterile Pasteur pipette.

Afterwards, the pellet was taken up in a total volume of 1 ml of cell medium. Then, 10 μl of the suspension was added to 90 μl 0.4% trypan blue (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and mixed thoroughly. A volume of 10 μl of this mixture was transferred into a Neubauer chamber. With a 10x objective one of the main squares is focused and viable and
dead (blue) cells are counted. Cells that touch the upper and left border are counted, cells that touch the right and lower border are excluded. The counting is performed in 4 main squares and the cell number of viable HIBCPP is averaged.

The actual amount of HIBCPP in the suspension was calculated for 1 ml according to formula 2.1 from chapter 4.1.3.

According to following procedures either a harvest to maintain the cell line until a maximum of 35 passages (application of 1-week culture or 2-week culture with 5x10⁶/cells or 1x10⁹/cells pro flask) was then carried out or a transfer to translucent ThinCert™Cell Culture Inserts for 24 well plates (membrane: 3 µm pore diameter) (Greiner Bio One International GmbH, Kreismünster, Austria) - hereinafter referred to as "filter" - was performed. For the former, the volume ($V_{Cells}$) to be supplemented into the T-75 flask was calculated using the following formula:

\[
V_{Cells} [ml] = \frac{\text{should number of cells [cells]}}{\text{have concentration [cells/ml]}}
\]

For seeding on filter, the cell suspension was adjusted to 1x10⁶ cells/ml. The appropriate addition of medium ($V_{Medium}$) was calculated using the formula 2.3:

\[
V_{Medium} [ml] = \left(\frac{\text{have concentration [cells/ml]}}{\text{should concentration [cells/ml]}}\right) - 1
\]

Afterwards the cells were harvested and 100 µl were transferred on turned around filter into a 12-well plate (Greiner Bio One International GmbH, Kreismünster, Austria) with an amount of 1x10⁵ cells per filter. The filters were totally filled obliquely from the downside with cell culture medium until a touch at the membrane was occurring. Subsequently the filters were moistened with 100 µl medium from the upside and were reincubated for the next 24 hours.

To prepare the anatomical correct alignment, the filter were flipped and transferred to 24 well plates (Greiner Bio One International GmbH, Kreismünster, Austria) for further growing the next 4 days to form a tight cell barrier with an upper “blood-compartment” and a lower “CSF-compartment” (figure 2.1). Volumes of the medium was always 1 ml in the lower part and 500 µl in the upper part. After 3 days, the medium was refreshed. One day before the infection
the filter were washed twice with DPBS and transferred into a new 24 well plate. The medium from this step on was free of antibiotics (1% FCS-medium).

Figure 16 The inverted transwell filter system for the HIBCPP
One day after seeding the HIBCPP on the membrane of a turned around filter, the filter is flipped. The cells are incubated in a hanging alignment for the next 4 days to form a tight barrier that separates the volumes of the well into a “blood compartment” and a “CSF compartment”. By this setup the correct in vivo alignment of the cells for infection is created.

4.1.5. Determination of barrier integrity

Barrier integrity was determined by measuring transepithelial electrical resistance (TEER) using the Millicell® ERS-2 (Merck Millipore, Billerica, USA) with the STX01 electrode to determine ohmic resistance between the upper and the lower compartment. Measurements were performed within 5 minutes after taking the samples out of the incubator and equilibrated electrodes in cell culture medium with a temperature of 37°C. The TEER of each filter was calculated by a multiplication of this value [Ω] with the area of the membrane [cm²] (Srinivasan et al. 2015). After having reached a TEER range between 360 – 550 Ωcm² the filter were used for infection studies. During the experiment with S. suis itself the TEER of the “infected” and “non-infected” filter was also determined at 2h and 6h p.i. (see chapter 4.1.7).

In addition, the flux of dextran with a molecular weight of 3000 (TexasRed®, ThermoFisher, Waltham, USA) through the cell barrier was determined for “infected” and “non-infected” filter with TEER ranges mentioned above. Therefore, a mixture of 5 µl dextran and 495 µl 1% medium was supplemented in the upper compartment after 2 hours post infection after removing remaining bacteria. For the “non-infected” filter the medium was replaced by this dextran mixture.
Four hours later the flux of dextran was determined by analysing the dextran amount in the lower compartment with TECAN Reader (Excitation 550 nm, Emission 620 nm, Gain 50%; infinite 200Pro; TECAN AG, Männedorf, Switzerland). The flux of dextran was calculated by comparison with a standard curve of known dextran concentrations. In case of the same concentration of dextran in the lower and the upper compartment the dextran flux was interpreted as 100 % (corresponding to a missing HIBCPP barrier on the filter). A concentration of 0 % dextran in the lower compartment was interpreted as a full stop of flux due to a fully tight barrier.

4.1.6. Determination of the cell number on filter with specified TEER range

The filter membranes of non-infected HIBCPP with specified TEER range of 360 – 550 Ωxcm² were cut out with a scalpel and put on microscope slides covered with 15 µl ProLong™ Gold Antifade Mountant including the DNA binding fluorescent stain 4′,6-Diamidino-2-phenylindol (DAPI) (ThermoFisher, Waltham, USA). Therefore, the nuclei were stained fluorescently and could be counted after excitation with ultraviolet light by the ApoTome (Carl Zeiss AG, Oberkochen, Germany; Objective: Plan-Apochromat 40x/0.95 Korr M27) in a gate of 0.0375 mm² (223.82 µm x 167.70 µm) in 3 different positions on the filter membranes (figure 2.2). The amount of cell nuclei was averaged and calculated up to the total membrane size with 33.6 mm².

Figure 17 Scheme for the determination of the amount of HIBCPP per filter

The cell nuclei were counted in 3 different positions on the filter membrane (a = 0.0375 mm²) and the results were averaged. Subsequently, the cell count was calculated for the entire size of the filter (a = 33.6 mm²).
4.1.7. Infection with *S. suis* and addition of human neutrophils

For the final experiment, the HIBCPP filter were transferred to 24-well SDR plates with integrated oxygen respectively pH sensors (chapter 3.1.7.2). The lower compartments were filled with 1 ml of 1% FCS- medium (chapter 4.1.4). Adapted to former studies (de Buhr et al. 2016) the upper compartments for “non-infected” HIBCPP filter were filled with 500 µl pathogen-free 1% FCS-medium whereas the upper compartment of “infection” filter was filled with 500 µl of a *S. suis* solution made of one of the cryostocks with a multiplicity of infection (MOI) of about 15 (CFU *S. suis*: 4.05x10^6 / HIBCPP on filter: 2.62x10^5). The experiments were performed four times at different days with changing *S. suis* batches (see chapter 4.1.1). The bacteria batches slightly differ in the concentrations of *S. suis* (C_{Bac}). In order to ensure a MOI of about 15 in each experimental run, the volumes (V_{Bac}) that have to be added from the stock (batch) into an “infection” filter were calculated according to the following formula 2.4:

\[
V_{Bac}[\mu l] = \left( \frac{4.05 \times 10^6 \text{CFU}}{C_{Bac} \text{[CFU/ml]}} \right) \times 1000
\]

As a comparative examination, 2.025x10^6 CFU of *S. suis* were directly added from the cryostock into wells filled with 1 ml 1% FCS-medium. The corresponding volume [µl] can be calculated by eq. 2.4 with a changed number of bacteria to 2.025x10^6 CFU. This number of bacteria correlates with the expected number of bacteria that transmigrate through the HIBCPP barrier and growth during an infection time of 2 hours.

All the samples were incubated for the next 2 hours.

Two hours post infection all upper compartments of the HIBCPP filter were refilled with pathogen-free 1% FCS-medium, partially added with 1.6x10^6 freshly harvested human neutrophils (PMN) (figure 2.3). In order to ensure a continuous ratio of about 6:1 (PMN: 1.62x10^6 / HIBCPP on filter: 2.62x10^5) the volumes (V_{PMN}) to be added from the different PMN suspensions were calculated according to the following formula 2.5:
Eq. 2.5 \[ V_{PMN}[\mu l] = \left( \frac{1.62 \times 10^6 \frac{PMN}{filter}}{C_{PMN} \frac{PMN}{ml}} \right) \times 1000 \]

Figure 18  **Infection procedure of HIBCPP cell model with following PMN transmigration**

*S. suis* was added to the basolateral side (blood compartment) of the HIBCPP with a MOI of 15. (A). The bacteria interact the next 2 hours with the HIBCPP and transmigrate into the lower compartment (CSF compartment) (B). After 2 hours, the remaining bacteria in the upper compartment are removed and replaced by a medium containing a PMN suspension for a PMN / HIBCPP ratio of 6:1. The PMN interact with the HIBCPP barrier and transmigrate into the CSF compartment (C). The oxygen in the lower compartment is measured using oxygen-sensitive sensors spots placed on the bottom of the well.

As a further comparative examination, 40% of the added PMN-volume \( (V_{PMN}) \) was supplemented directly into wells without HIBCPP but filled with 1 ml of 1% FCS-medium or bacteria (this corresponds to 20% of the amount of PMN added to the upper compartment of HIBCPP filter).

In addition, 2 \( \mu l \) of TNF\( \alpha \) (final concentration: 20 ng/ml) were added partially to the lower compartment of wells with or without HIBCPP.

The samples were incubated for the next 4 hours.

The exact assignment of the different wells with HIBCPP or without HIBCPP and their corresponding quantities of added 1% FCS-medium, *S. suis*, PMN or TNF\( \alpha \) is shown in figure 12 for the times 0 h, 2 h and 6 h post infection (p. i.). These setup times correspond to an host-pathogen interaction of 2 hours in the upper compartment, a corresponding transmigration time for the PMN of 4 hours into the lower compartment. Finally, after 6 hours of infection the investigation of the host-pathogen interactions inside the lower compartment can be performed. All samples were added in duplicates on the SDR “oxygen” plate, but only as a simple set on the SDR “pH” plate. In addition, a calibration solution with constant 0% oxygen saturation was measured in parallel as a technical control (chapter 4.1.9).
Table 12  Plate setup of the infection experiment with controls

Shown is the quantitative composition of the test samples and control samples for each well. The wells 1-6 are equipped with HIBCPP filters, the wells 8-11 are control samples without HIBCPP involvement. Wells 7 and 12 are used as technical controls for the oxygen measurement. Only the arrangement in well 6 mimics an infection setup to investigate host-pathogen interactions. Dashed boxes mark special supplements at appropriate times.
4.1.8. Determination of transmigrated S. suis and PMN

To monitor bacterial growth before and after the infection of the HIBCPP, colony forming units (CFU/ml) were determined after 0 (pre infection) and after 2 and 6 hours post infection. For the pre infection CFU determination bacterial samples were directly taken from the infection source (S. suis infection solution), for the post infection CFU, samples were taken from the lower compartment (“CSF compartment”).

Therefore 20 μl sample from the lower compartments (2 h and 6 h post infection) or from the S. suis infection solution (pre infection, 0 h post infection) were taken for each time after thorough homogenization of the sample with a pipette. The CFU/ml were determined according to chapter 4.1.2. For the pre infection CFU calculation, dilution series from $10^{-3}$–$10^{-5}$ were created, for the 2 h p.i. measurements the dilutions ranged between $10^{-1}$ – $10^{-4}$. The 6 h p.i. CFU/ml determination was performed with dilutions from $10^{-4}$ to $10^{-6}$.

The amounts of transmigrated PMN (transmigration was stimulated from the “CSF compartment” by S. suis or TNFα) after 4 hours of interaction with the HIBCPP barrier were determined by analysing samples from the lower “CSF compartment” by fluorescence-activated cell sorting (FACS; Attune NxT Flow Cytometer; life technologies Inc, Carlsbad, USA). Therefore, 1 ml volume was mixed with 333 μl of paraformaldehyde (PFA) 16% (Science Services GmbH, Munich, Germany) in 1.5 ml SafeSeal tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) to obtain a fixed sample with a final concentration of 4% PFA. The fixed samples were stored at +7°C. The analysis was based on Forward Scatter (FSC; detection of cell size) 180 and Side Scatter (SSC, detection of granularity) 350. The sample volume was 100 μl. The amount of PMN was calculated by gating for specific PMN characteristic. A characteristic FACS-plot is shown in figure 19. The left side (a) presents the plotting of transmigrated PMN after S. suis stimulation, the right side (b) shows a result of PMN after TNFα stimulus.

Due to technical losses of cells in the FACS device, the percentage of loss was determined with samples of known cell number. The results of counted PMN by the FACS device were corrected afterwards for the loss factor.
4.1.9. Non-invasive online oxygen and pH measurements in the infection model

In the study we used the SDR SensorDish® Reader Basis-Set (PreSens GmbH, Regensburg, Germany) in combination with one additional extension set. For parallel recording of oxygen and pH levels during the transmigration experiments, the selected HIBCPP filter were transferred to an OxoDish® OD24 or HydroDish® HD24 well plate (PreSens GmbH, Regensburg, Germany) and arranged according to the infection setup described in chapter 4.1.7.

All measurements were performed with equilibrated medium. Therefore, the respective wells in the plates were prefilled with medium incubated for at least 20 hours in the atmosphere of the incubator at 37°C (5% CO₂ and increased humidity due to H₂O evaporation of a water bowl). Wells without any oxygen consumers and equilibrated medium were used as positive controls (100% sat O₂). Wells with a negative control (0% sat O₂) were prepared as follows:

An amount of 1 g sodium sulfite (Na₂SO₃) and 50 μL cobalt nitrate (Co(NO₃)₂) of a standard solution (mass concentration (Co) = 1000 mg/L; in nitric acid 0.5 mol/L) were mixed. Afterwards, this mixture was dissolved in 100 ml normal water. The water becomes oxygen-free due to a chemical reaction of oxygen with Na₂SO₃. Additional oxygen, diffusing from air...
into the water, is removed by surplus of Na₂SO₃. Cobalt is used as catalyst to accelerate and complete the reaction of sulfite with oxygen.

All sensor plates were pre-calibrated by the company. The measurements were performed inside the cell incubator at 37°C. To maintain a continuous temperature of these 37°C during working steps outside the incubator (room temperature), all work was carried out on a 37 °C heating plate (CultureTemp®; Bel-Art Products-SP Scienceware, Wayne, USA).

The oxygen and pH calculations were performed with consideration of the temperature and the actual air pressure (for O₂ measurements) recorded directly in the incubator (barometer DK323 HumiBaroLog Plus; Driesen+Kern GmbH, Bad Bramstedt, Germany). The oxygen data were read out in the units % and mmHg (torr).

4.1.10. Influence of orbital shaking on the distribution of bacteria around the sensor spot

Since there are differences in the detected oxygen level in static and moving plate setups, the oxygen measurements were performed on orbital-shaked arrangements with 15 rounds per minute (rpm) (RS-OS 5; Phoenix Instrument GmbH, Germany). To monitor the bacterial distribution leading to a possible bacterial-provoked “microhypoxic” climate around the sensor area, two different setups were compared. One SDR plate with empty ThinCert™Cell Culture Inserts (filter) filled with 500 µl S. suis suspension was placed unmoved in the cell incubator, the other plate was orbitally shaked with 15 rpm. The bacteria suspension and quantification were performed as follows: S. suis ST2, strain 10 (chapter 3.2.1) was smeared on a Columbia agar plate with 6% sheep blood (Oxoid Deutschland GmbH) and was incubated for 16 – 18 hours at 37°C. After this time a single bacterial colony was picked and added into 10 ml Todd Hewitt Broth (THB) (Becton, Dickinson and Company, Sparks Glencoe, USA) in a
T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. The suspension was ice-stored incubated for 17 hours, leading to a delayed start of growth. After this incubation volumes of 500 µl S. suis suspension were added to empty filter for 24 well plates (Greiner Bio One International GmbH, Kreismünster, Austria). The well was filled with 1 ml 1% FCS-medium (see chapter 4.1.4). After 2 hours of incubation at 37°C and bacterial fall through the empty filter membrane, both plates were placed carefully in the refrigerator at 7°C to stop any further bacterial growth. Within the next 18 hours, the bacteria fell slowly to the bottom of the well, partially projecting the 3D-distribution gradient onto the surface. Afterwards, the medium was very carefully pipetted off and the bacteria in two defined areas (figure 20) were completely picked up with a sterile eyelet. The surface near the sensor spot ($A_1$) has been extended by a few millimetres to a defined radius of $r = 0.245$ cm and a surface of $0.19$ cm$^2$. The remaining surface of the outer ring ($A_2$) has therefore a size of $1.71$ cm$^2$ and is nine times bigger than $A_1$. To compensate for the different surface sizes for the CFU calculation, the eyelet samples were dissolved in different PBS volumes with a ratio of 1:9 ($V_1 = 0.5$ ml; $V_2 = 4.5$ ml). 2.1.2

To determine the CFU of $A_1$ and $A_2$ of both setups, 20 µl of each suspension was pipetted after thorough homogenization and used for the CFU/ml determination as described in chapter 4.1.2. The experiment was performed three times.

4.1.11. HIBCPP under hypoxic atmosphere of 3% O$_2$

To investigate the characteristics of the HIBCPP barrier and adaptations of the choroid plexus epithelial cells to hypoxic conditions the cells were incubated in a 3% O$_2$ atmosphere (37°C, 5% CO$_2$, humidified) in the “O$_2$ Control InVitro Glove Box” (Coy Laboratory Products, Inc; chapter 3.1.8).

Therefore, HIBCPP filter (primary incubated under normoxic atmosphere of 18.5% O$_2$) with defined TEER ranges between 360 – 550 Ωxcm$^2$ were investigated. The HIBCPP filter were transferred to 1 ml equilibrated 1% FCS-medium (see chapter 4.1.4) and were filled with 500 µl S. suis suspension, corresponding to a MOI of 15 (see chapter 4.1.7) or with pathogen-free cell culture medium. The HIBCPP barrier integrity was determined by TEER measurements and
dextran flux according to the procedure described in chapter 4.1.5. The measurements were performed after 2 and 6 hours of hypoxic incubation in 3% O₂ atmosphere.

In parallel, the same experiment was also performed under normoxic condition with 18.5% O₂. The incubations were performed in duplicates. Next to the individual samples, pooled samples were prepared. Table 13 gives an overview about the respective samples and the concentration of cDNA of the pooled samples (chapter 4.1.11):

<table>
<thead>
<tr>
<th>Oxygen atmosphere</th>
<th>Hypoxia (3% O₂)</th>
<th>Normoxia (18.5% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIBCPP</td>
<td>HIBCPP</td>
</tr>
<tr>
<td>infection status</td>
<td>non infected</td>
<td>S. suis</td>
</tr>
<tr>
<td>incubation time</td>
<td>2 h</td>
<td>6 h</td>
</tr>
<tr>
<td>samples</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>pooling</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>cDNA concentration [ng/µl]</td>
<td>49.6</td>
<td>61.4</td>
</tr>
</tbody>
</table>

Table 13 Overview of the normoxic and hypoxic incubated HIBCPP
The HIBCPP were incubated under different oxygen atmospheres and infection status. Shown is also the scheme of pooling the samples with respective cDNA concentration

4.1.12. RNA profile - expression of vegf and the housekeeping gene rsp9
To monitor differences in cell responds to different oxygen atmospheres the cells were incubated either under normoxic conditions (18.5% O₂) or hypoxic conditions (3% O₂) for 2 or 6 hours. Additionally, the cells were infected or stayed without bacterial contact (chapter 4.1.11).

For the RNA preparation the RNeasy Mini Kit (Qiagen N.V., Venlo, Netherlands) was used according to the manufacturer's instructions. Therefore, the HIBCPP filter were washed twice with Dulbecco’s Phosphate Buffered Saline (DPBS; ThermoFisher, Waltham, USA) and were placed cell side down in a well of a 12-well plate (Greiner Bio One International GmbH, Kreismünster, Austria) filled with 350 µl RLT buffer (component of the kit). To disrupt the cells and plasma membranes the filters were rubbed on the bottom for half a minute. In addition,
the filters were rinsed twice with this well-filled RLT buffer with a pipette. The complete
volume was then supplemented with 3.5 µl of 2-mercaptoethanol (Carl Roth GmbH + Co. KG,
Karlsruhe, Germany) and homogenized by pipetting up and down 10 times with a 1 ml syringe
(Inject®-F Solo, B. Braun Melsungen AG, Germany) and 20G cannula (Sterican® Gr. 1, G 20 x 1
1/2” / ø 0,90 x 40 mm, B. Braun Melsungen AG, Germany). The following steps of the RNA
extraction are in accordance with the manufacturer's protocol but were supplemented with a
one time on-column DNase treatment to additionally digest DNA during the RNA purification.
Therefore, the RNase-Free DNase Set (Qiagen N.V., Venlo, Netherlands) was used according
to the manufacturer’s protocol.

After RNA extraction the concentration and purity of the isolated RNA was determined by
using the Agilent 2100 bioanalyzer (RNA 6000 Pico Kit; Agilent Technologies Inc., Santa Clara,
USA). Only samples with RNA integrity number (RIN) values over 8.2 were used for further
steps.

With the TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany) the RNA
samples were reversely transcripted into cDNA by using Invitrogen® Random Hexamers (5 nM;
ThemoFisher, Waltham, USA), Invitrogen® dNTP Set (10mM; ThemoFisher, Waltham, USA)
and M-MLV Reverse Transcriptase + M-MLV RT 5X Buffer (Promega, Madison, USA). The
conducted thermoprofile was 70 ºC for 10 minutes, followed by a pause on ice (for further
working steps) and ended by incubation at 42ºC for 1 hour with a short final heat incubation
for 5 minutes at 85°C. Transcription was performed with negative controls (-RT) and no
template controls (NTC).

Afterwards, a real-time PCR (qPCR) was performed to analyse the expressions of vegf (chapter
3.1.5) and rps9 (housekeeping gene encoding for a human ribosomal protein known to be an
unregulated gene (Eisenberg and Levanon 2003)). The respective primers (100 µM; Sigma
Aldrich, St. Louis, USA) are given in table 14.
Table 14  Primer sequences for vegf and rps9
The sequences of vegf and the human housekeeping gene rps9 with their corresponding amplicon lengths

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′) forward (vw)</th>
<th>Primer sequence (5′-3′) reverse (rev)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vegf</td>
<td>ATGAACTTTCTGCTGTCTTGGGT</td>
<td>TGGCCTTTGGTGAGGGTTGGATCC</td>
<td>344</td>
</tr>
<tr>
<td>rps9</td>
<td>CTGACGCTTTGATGAGAAGGAC</td>
<td>CTCATCCAGCACCCCAAT</td>
<td>87</td>
</tr>
</tbody>
</table>

To measure the cycle thresholds (Ct) for vegf and rps9 from the different HIBCPP samples, the following reagents were prepared:

First, a 10 µM “VEGF working solution” was prepared by mixing 10 µl VEGF<sub>fw</sub> + 10 µl VEGF<sub>rev</sub>+ 80 µl nuclease-free water (Sigma Aldrich, St. Louis, USA). The same was done for the RPS9 primer. To create the “master-mixes”, volumes of 1 µl of the “working solutions” were supplemented each with 10 µl of QuantiTect SYBR® Green PCR Master Mix (Qiagen N.V., Venlo, Netherlands) and 4 µl nuclease-free water. Therefore, each master-mix had a volume of 15 µl in total. Each “master-mix” was added to 5 µl of the respective cDNA samples and the qPCR was conducted as previously described (Branitzki-Heinemann et al. 2016; J. Willenborg et al. 2011) with the following modified program in the AriaMX Real-Time PCR system (Agilent Technologies Inc., Santa Clara, USA):

- Initial denaturation at 95°C for 20 min
- 40 cycles of denaturation at 95°C for 25 s
- Annealing at 58°C for 30 s
- Amplification at 72°C for 20 s using

The resulting amplicon products were verified by melting curve analysis and by visualization with a 120 V gel electrophoresis for 90 minutes (1.5% agarose, Universal-Agarose (peqGOLD); VWR, Radnor, USA) according to general practice. Fragment sizes were determined by comparison to GeneRuler 50 bp DNA Ladder fragments (ThermoFisher, Waltham, USA).

For the calculation of the primer efficiencies on the target gene sequences for VEGF and RPS9 the cDNA was pooled for normoxic “non-infected” and “S. suis” samples such as for hypoxic “non-infected” and “S. suis” samples. The concentration of cDNA of the pools was measured.
with Multiskan GO (ThemoFisher, Waltham, USA) according to the manufacturer's instructions.

To determine the efficiencies of the primers the C_{t} values for different templet volumes of the pooled samples are needed. Therefore, the templet volumes were 9 µl – 7.5 µl – 5 µl – 3.5 µl – 2 µl and 0 µl. Reduced or increased template volumes have been compensated with nuclease-free H_{2}O. The qPCR was performed as described before.

The measured C_{t} values were displayed with the associated logarithmical template quantities (cDNA [ng/µl] x templet [µl]; see table 13) on a X-Y-plot.

Next, linear regression curves through the data points were generated to calculate the slopes of the different trend lines. This was performed using the statistical software GraphPad Prism 8.0. Finally, primer efficiency is calculated by equation 2.7:

\[
E = -1 + 10^{\frac{-1}{\text{slope}}}
\]

To compensate for possible technical inaccuracies due to the small pipetting volumes, the primer efficiencies were determined in 4 experiments and the results were averaged. Possible outliers were identified with the Grubbs’ outlier test (α=0.1, GraphPad Prism 8.0) and were excluded for the calculation.

To finally determine the relative transcription rates (R, ratio) of vegf under hypoxia or under infection in compliance to the primer efficiencies, the calculation according to Souazé et al. (Souazé et al. 1996) was performed. Possible outliers of the individual sample C_{t} were previously identified with the Grubbs’ outlier test (α=0.1, GraphPad Prism 8.0) and were not included in the calculation.

In consideration of the housekeeping gene RSP9 and the primer efficiencies, the formula to calculate the expression of vegf in hypoxia in relation to normoxia is shown in equation 2.6:

\[
R = \frac{(E_{\text{rsp9, hypoxia}})^{C_{\text{rsp9, hypoxia}}}}{(E_{\text{vegf, hypoxia}})^{C_{\text{vegf, hypoxia}}}} / \frac{(E_{\text{rsp9, normoxia}})^{C_{\text{rsp9, normoxia}}}}{(E_{\text{vegf, normoxia}})^{C_{\text{vegf, normoxia}}}}
\]
4.2. In vivo: Studies on living pigs

4.2.1. Stabling and handling of the animals

At least one week before the start of the experiment, piglets with an initial body weight of 12.35 ± 1.65 kg were stabled in groups in the facilities of the Research Center for Emerging Infections and Zoonoses (RIZ) of the University of Veterinary Medicine Hannover. The piglets used in this study are German hybrid breeds from the “Bundeshybridzuchtprogramm” (BHIZP). The piglets are produced by mating the BHIZP hybrid sow "db.Victoria®“ (parent breeds: German Large White (sow) + German Landrace (boar)) with the boar “db.77®” from the Piétrain family.

The experiment was divided into 3 runs (R) with R1=3, R2=5 and R3=6 animals. The study was statistically calculated for 12 animals in total, 6 got infected, 6 functioned as controls. Two more piglets were additionally stabled. These animals functioned as back-up animals to prevent housing of a single animal left over at the end of the test series (ensuring the animal-specific herd instinct) or for replacing a pig in case of a failed infection experiment.

In total there were 8 females, 2 males and 2 castrated piglets used in the experiments. Table 15 gives an overview about the individuals:

<table>
<thead>
<tr>
<th>Experimental No.</th>
<th>Gender</th>
<th>Run</th>
<th>S. suis (CFU/ml) or PBS</th>
<th>Age (days) at day of infection</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>W</td>
<td>1</td>
<td>PBS</td>
<td>51</td>
<td>162</td>
</tr>
<tr>
<td>H2</td>
<td>W</td>
<td>1</td>
<td>3.11x10^8</td>
<td>52</td>
<td>162</td>
</tr>
<tr>
<td>H3</td>
<td>W</td>
<td>1</td>
<td>3.75x10^8</td>
<td>54</td>
<td>162</td>
</tr>
<tr>
<td>H4</td>
<td>W</td>
<td>2</td>
<td>PBS</td>
<td>47</td>
<td>218</td>
</tr>
<tr>
<td>H5</td>
<td>M</td>
<td>2</td>
<td>PBS</td>
<td>48</td>
<td>218</td>
</tr>
<tr>
<td>H6</td>
<td>M</td>
<td>2</td>
<td>2.75x10^8</td>
<td>50</td>
<td>218</td>
</tr>
<tr>
<td>H7</td>
<td>W</td>
<td>2</td>
<td>2.54x10^8</td>
<td>54</td>
<td>218</td>
</tr>
</tbody>
</table>
During the period of group housing, the animals were fed twice a day with piglet feed. Water was available *ad libitum* via nipple drinkers. The animal husbandry complied with the legal requirements for animal welfare of the European Union and German laws and was approved by the authorities under the reference number 33.8-42502-04-18/2879 (LAVES; Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Germany). The pigs were checked twice per day for well-being. The stables were equipped with different toys for activity like teething rings, cords and balls. The animals were playfully accustomed to rectal fever measurement and other procedures to minimize stress-associated disorders like body temperature increases in time of important measurement processes during the concrete experiments.

### 4.2.2. Preliminary trainings for the CSF puncture at the atlantooccipital joint

In order to get good puncture results as fast as possible of the CSF at the atlantooccipital joint in living animals, the procedure of finding the correct puncture site and insertion of a catheter into the subarachnoid space was preliminary trained on half-carcasses at the *Institute for Food Quality and Food Safety* (TiHo) (figure 21) and on dead pigs of the same weight class by Computed Tomography (CT) control at the *Clinic for small mammals, reptiles and birds* (TiHo) (figure 22).
4.2.3. Preparation of the *Streptococcus suis* solution for injection

From a -80 °C frozen cryostock *S. suis* serotype 2 strain 10 (chapter 3.2.1) was smeared on a Columbia agar plate with 6% sheep blood and incubated for approximately 20 hours in an incubator at 37°C without CO₂ supplementation. After this time two bacterial colonies were picked and added into 10 ml Tryptic Soy Broth without dextrose (TSB) (Becton, Dickinson and
Company, Sparks Glencoe, USA) filled in T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. After 10 hours of further incubation (here: 37°C / 5% CO₂) a 1:100 dilution was created in an Erlenmeyer flask with preheated TSB followed by a short incubation phase (again: 37°C / 5% CO₂) of about 3 hours. Towards the end of the 3-hour incubation, the optical density in the Erlenmeyer flask was determined regularly by photometry at 600 nm (OD₆₀₀) to exactly get the OD 0.3 ±0.02. At this time 40 ml from the bacterial culture were transferred to a 50 ml Falcon Tube (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and were centrifuged at 4816 g for 10 minutes (Heraeus Multifuge X3R centrifuge, Thermo Fisher Scientific Inc, Waltham, USA) at room temperature. The supernatant was discarded, and the pellet was resuspended in 3 ml sterile phosphate-buffered saline (PBS). From this suspension a 1:10 dilution was made. This was used for the inoculation to infect the pigs and for the following determination of the concrete infection dose. For the latter, a volume of 20 μl of the pre-diluted suspension was used to create further dilutions up to 10⁻⁷ according to chapter 4.1.2. Dilutions 10⁻⁴ to 10⁻⁷ were plated on the agar plates. After incubation a CFU/ml of about 1 x 10⁻⁸ was expected.

4.2.4. Determination of hygiene status pre-infection and inoculation of the bacteria

For the infection experiments, the respective pig was briefly anesthetized by intramuscular injection in the Musculus (M.) biventer cervicis near the ear basis with a 21 G cannula (Sterican 0.80 x 40 mm, B. Braun Melsungen AG, Germany). The drug was of a combination of ketamine (20 mg/kg) and azaperone (2 mg/kg).

In the state of general anaesthesia, a tonsillar swab (Amies medium, SARSTEDT AG & Co. KG, Nümbrecht, Germany) was taken and blood from the Vena (V.) cava cranialis was withdrawn into serum and plasma (lithium-heparin) monovettes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) with 20 G or 18 G cannula (Sterican 0.9/1.2 x 40 mm, B. Braun Melsungen AG, Germany) after disinfection of the puncture site with alcohol swabs. For further handling of the swab or the blood sample see chapters 4.2.8 and the followings.

After these initial probe samplings 1 ml of the S. suis inoculum (effective infection dose between 2.5-3.7x10⁸ CFU/ml) or phosphate-buffered saline (PBS) as control medium was injected via the ear vein with a 21 G cannula (Sterican 0.80 x 25 mm, B. Braun Melsungen AG, Germany) after disinfection of the puncture site with alcohol swabs.
After the infection the animal was housed separately for the next 8-12 hours and was closely monitored starting 8 hours post-infection for any symptoms of infection by means of a score (see appendix). The recovery phase took place under observation. In case of reaching a score of 25 early, general anaesthesia was initiated immediately for reasons of animal welfare. Therefore, the piglet was undergoing under anaesthesia control and monitoring (chapter 4.2.5). Otherwise, the start of anaesthesia was started regularly 12 hours post infection. The experiment was carried out on piglets at the age between 47 and 62 days of life (7-9 week).

4.2.5. Long-term anaesthesia and control of physiological parameter

Twelve hours post infection (or earlier in case of a score of 25) the infected pigs (or control animals) were anaesthetized by intramuscular injection of a combination of ketamine (20 mg/kg), azaperone (2 mg/kg) and atropine (0.06 mg/kg) as anticholinergic medication.

Venous access was established by an indwelling venous catheter (Vasovet Braunüle, 20G/22G; B. Braun Melsungen AG, Germany) either in an ear vein, the cephalic or lateral saphenous vein. Anaesthesia was deepened by the injection of propofol (1-2 mg/kg) until endotracheal intubation was possible. After topical anaesthesia with tetracaine spray (2-3 sprays of about 0.7 mg tetracaine base), the pigs were endotracheally intubated under visual control with a cuffed endotracheal tube (Portex 100/150/XX ID 5-6; Smiths Medical, Inc., Minneapolis, USA). The endotracheal tube was connected to a circle breathing system (DRAEGER Titus; Drägerwerk AG & Co. KGaA., Lübeck, Germany) and volume controlled mechanical ventilation (DRAEGER Ventillog C; Drägerwerk AG & Co. KGaA., Lübeck, Germany) with a tidal volume of 15-20 ml/kg, a respiratory rate of 20/min and a pressure limitation of 20 mbar was immediately started and adjusted to maintain eucapnia. The anaesthesia was maintained with isoflurane (Isofluran CP 1 ml/ml, 250 ml, CP-pharma; CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany) in air, supplemented with oxygen if needed. The FiO₂ was adjusted to maintain a physiological arterial partial pressure of oxygen of 80 to 110 mmHg and to avoid hyperoxia. Isoflurane anaesthesia was supplemented by a constant rate infusion of ketamine (1 mg/kg/h) and dexmedetomidine (2 µg/kg/h) and 2 boli of levomethadone/fenpipramide (L-Polamivet 2,5/0,125 mg/ml®, Intervet GmbH, Unterschleißheim, Germany) were injected intravenously immediately after intubation and after 4 hours of anaesthesia (0.15 mg/kg).
The depth of anaesthesia was monitored by testing muscle relaxation, the eye reflexes, the interdigital withdrawal reflexes and skin stimulation with an artery clamp type Kelly. The ECG, heart rate, arterial blood pressure, peripheral oxygen saturation (SrO₂) via transmission pulse oximetry at the tail or a claw, end-tidal CO₂, inspired and expired isoflurane concentrations and the rectal temperature were continuously monitored via an anaesthesia multiparameter monitor (DATEX Cardiocap 5, General Electric Company, Boston, USA). For intravascular blood pressure measurement access was made to the femoral artery by an arterial catheter (BD Insyte-W, 22G; Becton Dickinson, Franklin Lakes, USA). The pressure was recorded via a precalibrated pressure transducer (BRAUN Combitrans Monitoring set venous; B. Braun Melsungen AG, Germany) levelled and zeroed to ambient pressure at the base of the heart.

To control physiological blood parameters arterial blood gas measurements were performed using the EPOC system (epoc® Blood Analysis System; Siemens Healthcare GmbH, Erlangen, Germany) before each oxygen measurement in the CSF and individually as needed for anaesthesia management to maintain an adequate depth of anaesthesia. Mean arterial blood pressure was maintained above 60 mmHg by fluid infusion and inotropes or vasopressors as required.

Table 16 lists the drugs that were used for each individual animal if needed to ensure effective anaesthesia.

<table>
<thead>
<tr>
<th>substance</th>
<th>dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction of long-term anaesthesia</td>
<td></td>
</tr>
<tr>
<td>propofol</td>
<td>1-2 mg/kg IV</td>
</tr>
<tr>
<td>Constant rate infusion (CRI): 5 ml/kg/h</td>
<td></td>
</tr>
<tr>
<td>Sterofundin ® 1/1 E (B. Braun Melsungen AG, Germany)</td>
<td></td>
</tr>
<tr>
<td>dexmedetomidine</td>
<td>2 µg/kg/h</td>
</tr>
<tr>
<td>ketamine</td>
<td>1 mg/kg/h</td>
</tr>
<tr>
<td>dopamine</td>
<td>5-10 µg/kg/min</td>
</tr>
<tr>
<td>noradrenaline</td>
<td>0.1-0.25 µg/kg/min</td>
</tr>
<tr>
<td>dobutamine</td>
<td>3-5 µg/kg/min</td>
</tr>
<tr>
<td>additional medication</td>
<td></td>
</tr>
<tr>
<td>levomethadone/fenpipramide</td>
<td>0.15 mg/kg IV</td>
</tr>
<tr>
<td>(L-Polamivet® 2,5/0,125 mg/ml)</td>
<td></td>
</tr>
</tbody>
</table>

Table 16  Overview of drugs used during anaesthesia
The temperature of the animal was maintained by means of red-light heat lamps and adjustable electrical heating mats over the period of anaesthesia. Initial temperature was the temperature at the time of induction of anaesthesia. After completion of the measurements, the animals were euthanized intravenously with T 61 ® (Intervet Deutschland GmbH, Unterschleißheim, Germany) with 3-4 ml/50 kg during isoflurane anaesthesia for further examination.

4.2.6. Oxygen and pH measurements in the CSF

Following intubation, the pig's neck area was shaved around the Articulatio atlantooccipitalis (atlantooccipital joint). The neck was moved and the point of most flexibility was marked with a line. The animal was placed in lateral recumbency and the neck was bent at maximum towards the rib cage and fixed by tension straps. Afterwards the head was lined with towels until it was positioned exactly horizontal to the table. After disinfection of the puncture site (approximately 1 cm caudal the marked line), a Spinocan® epidural needle (1,30 x 88 mm, G 18 x 3 1/2”; B. Braun Melsungen AG, Germany) was inserted in rostral direction. After a slight resistance by penetrating the arachnoid membrane the stylet was removed. In best case clear CSF was immediately dripping from the needle as a sign of entering the subarachnoid cavity. If no CSF was observed or in case of blood contamination the needle was slightly changed in position until clear CSF was flowing out. The needle was then connected to the 10 cm long tube of a three-way valve (Discofix®-C; B. Braun Melsungen AG, Germany) connected to a pressure transducer (Combitrans ®; B. Braun Melsungen AG, Germany). The FTC oxygen sensor (FTC-Pst7; PreSens Precision Sensing GmbH, Regensburg, Germany) and the pH sensor (FTC-SU-HPS-US; PreSens Precision Sensing GmbH, Regensburg, Germany) were then connected in series to the leftover connection. By the open side of the pH sensor it was possible to collect CSF samples with a syringe by a second 3-way valve (figure 23).
Figure 23  **Setup for oxygen and pH measurements in the CSF**

The catheter is continuously installed in the CSF and connected via 3-way valves with the FTC sensors for oxygen and pH measurements. If the 3-way valves are set accordingly, CSF can flow via route B into the FTC sensors and CSF samples can be obtained afterwards or the pressure of the CSF (pCSF) can be monitored continuously via route C. For rinsing procedures, route A is important.

After installation of the measurement setup, the 3-way valve was open that way that the pressure of the CSF (pCSF) was permanently monitored. The pressure measurement for the CSF was performed as for the blood pressure (chapter 4.2.5). Initially, 5 minutes before the first O₂ measurement, the three-way valves were opened that way that 1 ml of CSF was drawn into a 2 ml syringe via the oxygen and pH probes connection. Thereafter, the three-way valves were reversed so that CSF was trapped over the sensors without any air contact. For the next 5 minutes the sensor probes equilibrated to the CSF. The CSF in the syringe was disposed. After this pre-treatment, the concrete measurement was carried out regularly 1 hour after starting the isoflurane anaesthesia (13 hours post infection), if possible. To do this, the three-way valves were turned again so that 1 ml of sterile cerebrospinal fluid could be drawn into a new 2 ml syringe via the oxygen and pH sensors. The CSF sample in the syringe was transported on ice to the laboratory for further examinations (chapter 4.2.8.2). The three-way valves were closed again so that CSF was trapped over the measurement sensors without having air contact. The measuring sensors were covered with aluminium foil to keep the temperature constantly and to protect the sensors from light. The measuring was performed every 1 minute over a time span of 10 minutes in total. The total pressure for the calculation was the prevailing air pressure (pₐₐᵢₙ) of the room (measured with Fisherbrand™ Traceable Digital Barometer; Thermo Fisher Scientific Inc, Waltham, USA) in addition to the CSF pressure measured directly before the three-way valves were opened. Since an exact temperature
measurement of the CSF is not possible, the rectally measured body temperature was taken as the basis for the $O_2$ calculation. For pH measurements the air pressure compensation is not needed. While the pH measurement shows relatively stable measurements over the 10-minute measurement period, the oxygen measurement results in a characteristic curve due to equilibration and temperature effects. The oxygen measurement result for the experiment was defined as the lowest measurement point during the measurement period (figure 24).

![Typical oxygen curve during measurements](image)

<table>
<thead>
<tr>
<th>t [min.]</th>
<th>$pO_2$ [mmHg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>57.3</td>
</tr>
<tr>
<td>1</td>
<td>53.0</td>
</tr>
<tr>
<td>2</td>
<td>52.5</td>
</tr>
<tr>
<td>3</td>
<td>52.9</td>
</tr>
<tr>
<td>4</td>
<td>53.6</td>
</tr>
<tr>
<td>5</td>
<td>54.3</td>
</tr>
<tr>
<td>6</td>
<td>55.1</td>
</tr>
<tr>
<td>7</td>
<td>55.9</td>
</tr>
<tr>
<td>8</td>
<td>56.8</td>
</tr>
<tr>
<td>9</td>
<td>57.8</td>
</tr>
<tr>
<td>10</td>
<td>58.7</td>
</tr>
</tbody>
</table>

Figure 24 Curve of a typical single oxygen measurement with the FTC sensor

The measurement of a CSF sample does not immediately yield a result but shows a curve-like course due to various physical influences. The final measurement result is defined as the lowest point of the curve over a measurement interval of 10 minutes.

After the measurements were performed, route A of the measurement setup (figure 23) was rinsed with 5 ml of the infusion medium (Sterofundin ®) via the way of the pressure transducer. By reorienting the respective three-way valves, the continuous measuring of the CSF pressure was restarted afterwards. After 4 and 7 hours after the start of isoflurane anaesthesia (means 16 h p.i. / 19 h p.i.), the oxygen and pH measurements in the CSF were repeated. For this purpose, the route B was rinsed first to purify from any bacterial contamination left over from the measurement periods before by drawing 1.8 ml of new CSF into a syringe with appropriate orientation of the three-way valves. This probable contaminated CSF was then discarded. Before the defined measurements, a 5-minute adaptation phase of the measuring sensors was again carried out, as described for the first measurement. The actual measurements were done accordingly.
4.2.7. Influence of blood oxygenation on the oxygen level in CSF

To check whether hypo- or hyperoxygenation of the blood caused by artificial ventilation alters the oxygen content in the CSF, two special experiments were carried out. First the animal was ventilated with 100% oxygen for 35 minutes from the condition of stable, physiological blood oxygenation and corresponding oxygen value in the CSF. Subsequently, the oxygen content in the blood and in the CSF was determined in order to detect possible changes to the physiological blood oxygenation.

The influence of a possible hypoxigination on the CSF oxygen content was simulated by a cardiac arrest with stop of the blood circulation and thus suppression of the continuous oxygen supply in the organism (heart rate: 0/min). For this purpose, the oxygen level in the CSF was measured in the living animal after a long-lasting phase (at least 3 hours) of physiological blood oxygenation. The animal was then euthanized. 10 minutes after the cardiac arrest had been detected, the oxygen level in the CSF was again measured via the CSF catheter.

Blood gas measurements and oxygen measurements were made as previously described.

4.2.8. Handling of blood, CSF and autopsy samples obtained in the animal experiments

Before the inoculation of S. suis or PBS (see chapter 4.2.3) into the pig blood from the Vena (V.) cava cranialis was filled into serum and plasma (lithium-heparin) monovettes (SARSTEDT AG & Co. KG, Nümbrecht, Germany). During isoflurane anaesthesia, these blood samples were also collected via indwelling venous catheter (or via the arterial access). The blood samples were taken at 13, 16 and 19 hours after infection. In parallel a sample of 1-2 ml fresh CSF was collected via the CSF catheter if possible. It was ensured that the serum samples stood upright for 30 minutes after time of collection before further working steps. Immediately after the collection the serum, plasma and CSF samples were transferred on ice to the laboratory for the following procedures:
4.2.8.1. Processing of the blood samples

First, from the post infection lithium-heparin samples a volume of 200 μl was removed for the determination of colony forming units, for testing for pure \textit{S. suis} culture and further molecular investigations. The rest was centrifuged at 4°C for 10 minutes at 2100 g (Heraeus™ Multifuge™ X3; ThermoFisher, Waltham, USA). The supernatant was aliquoted and flash frozen in liquid nitrogen. Long-time storage is at -80 °C.

From the 200 μl aliquot a volume of 20 μl coagulation inhibited blood was pipetted and smeared fragmentally on Columbia agar plate with 6% sheep blood (Oxoid Deutschland GmbH). Therefore, a sterile eyelet was used. After 24 hours of incubation at 37°C the smear was checked for pure \textit{S. suis} culture by morphology (e.g alpha-hemolyzing colonies).

A further volume of 20 μl was used for the determination of colony forming units (CFU/ml) according to chapter 4.1.2. Dilution series up to 10^{-5} were plated for animals suffering from infection symptoms (body temperature > 41°C, CNS symptomatic or turbid CSF), for control animals or animals without infection symptoms dilutions up to 10^{-2} were plated. In contrast to the method described in chapter 4.1.2 two dilutions were plated on one plate. The blood agar plates were incubated at 37°C for the next 24 hours.

A volume of 100 μl from the 200 μl aliquot is transferred into 5 ml Todd Hewitt Broth (THB) (Becton, Dickinson and Company, Sparks Glencoe, USA) in a T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. The following incubation was 12-24 hours at 37°C.

Afterwards a fragmented smear (20 μl) with a sterile eyelet was created from this culture on a Columbia agar plate with 6% sheep blood to check again for pure \textit{S. suis} culture by morphology after 24 hours of incubation at 37°C.

The rest of the sample was centrifuged at 2600 g for 5 minutes. The resulting bacterial pellet was stored at -20 °C for DNA examination.

The serum blood samples were centrifuged at 2100 g for 10 minutes at 4°C after a standing time of 30 minutes. The supernatant was aliquoted and flash frozen in liquid nitrogen. Long-time storage is at -80 °C.
## 4.2.8.2. Processing of the CSF samples

For the quantification of PMN in the cerebrospinal fluids volumes of 200 µl CSF were first supplemented with paraformaldehyde (PFA) (Science Services GmbH, Munich, Germany) to a final concentration of 4% for fixation in 1.5 ml SafeSeal tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany). The samples were short-time stored at +4°C and later analyzed together by fluorescence-activated cell sorting (FACS; Attune Nxt Flow Cytometer; life technologies Inc, Carlsbad, USA). The analysis was based on Forward Scatter (FSC-A; detection of cell size) 180 and Side Scatter (SSC-A, detection of granularity) 350. The sample volume was 100 µl. The number of neutrophil granulocytes was calculated by gating for specific PMN morphology. In addition, the total number of cells and the number of singlets was determined (using FFS-A versus FFS-H scatter).

For microscopic determination of the cells in the CSF, 50 µl of sample was applied to a standard slide and spread over a small area with a sterile loop. Subsequently, the smear was air-dried and a Diff Quick staining (HAEMA SCHNELLFÄRBUNG Diff-Quick, LT-Sys ®, Berlin, Germany) was performed according to the manufacturer’s instructions. The stained samples were then examined microscopically (BX81, Olympus, Tokyo, Japan) to differentiate morphologically different cell types in the CSF. To counteract the altered morphology of the cells by the process of dehydration, the samples were wetted with Roti®-Mount (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) immediately prior to microscopic examination and covered with a coverslip.

A volume of 20 µl CSF was smeared fragmentally on a Columbia agar plate with 6% sheep (Oxoid Deutschland GmbH). Therefore, a sterile eyelet was used. After 24 hours of incubation at 37°C the smear was checked for pure S. suis culture by morphology (e.g alpha-hemolyzing colonies).

A further volume of 20 µl was used for the determination of colony forming units (CFU/ml) according to chapter 4.1.2. Dilution series up to 10^-5 were plated for animals suffering from infection symptoms (body temperature > 41°C, CNS symptomatic or turbid CSF), for control animals or animals without infection symptoms dilutions up to 10^-2 were plated. In contrast to
the method described in chapter 4.1.2 two dilutions were plated on one plate, except for the $10^{-5}$ dilution. The blood agar plates were incubated at 37°C for the next 24 hours.

A volume of 100 µl CSF was transferred into 5 ml Todd Hewitt Broth (THB) (Becton, Dickinson and Company, Sparks Glencoe, USA) in a T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. The following incubation was 12-24 hours at 37°C. Afterwards a fragmented smear (20 µl) with a sterile eyelet was created from this culture on a Columbia agar plate with 6% sheep blood to check for pure *S. suis* culture after 24 hours of further incubation.

In case of turbidity of the THB medium, the suspension was centrifuged at 2600 g for 5 minutes. The resulting bacterial pellet was stored at -20 °C for DNA examination.

All leftover CSF on the day of the experiment was aliquoted and flash frozen in liquid nitrogen. The temperature for long-time storage is at -80 °C.

### 4.2.8.3. Processing of the autopsy samples

After completion of the respective measurements on the living animal, the pig was euthanized for animal welfare reasons in state of general anaesthesia by applicating 3-4 ml of T 61 ® (Intervet Deutschland GmbH, Unterschleißheim, Germany) intravenously. After death was determined by means of electrical monitoring and auscultation, necropsy was performed to examine certain organs macroscopically for signs of infection. Different samples were taken for the microbiological follow-up examination or histopathological examination. For the latter, the samples were embedded directly in 10 % formalin. Table 17 provides information on the organ specific samples.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Microbiological sample</th>
<th>Histopathological sample (formalin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (Lobus quadratus)</td>
<td>1.5 x 1.5 cm</td>
<td>2.0 x 3.0 cm</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.5 x 1.5 cm</td>
<td>2.0 x 3.0 cm</td>
</tr>
<tr>
<td>Lung (Lobus cranialis sinister)</td>
<td>2.0 x 3.0 cm</td>
<td>2.0 x 3.0 cm</td>
</tr>
<tr>
<td>Tonsil</td>
<td>sterile swab(^1) (pre-infection)</td>
<td>1.0 x 1.0 cm</td>
</tr>
<tr>
<td>Peritoneum</td>
<td>sterile swab(^1)</td>
<td>2.0 x 3.0 cm</td>
</tr>
<tr>
<td>Pleura costalis</td>
<td>sterile swab(^1)</td>
<td>2.0 x 3.0 cm</td>
</tr>
<tr>
<td>Pericardium</td>
<td></td>
<td>Ventriculus cordis sinister</td>
</tr>
<tr>
<td>Heart (Valva atrioventricularis sinister)</td>
<td>sterile swab(^1)</td>
<td></td>
</tr>
</tbody>
</table>
Table 17 Overview of organ samplings for S. suis detection

For the microbiological examination, the organ pieces were placed in plastic dishes and were undergoing the following procedure:

The organ pieces were dipped in 100% alcohol (except lung) and were briefly flamed. With flamed, sterile scissors and tweezers a fresh cut was generated and pressed 2-3 times for 1-2 seconds on a Columbia agar plate with 6% sheep blood. About 1/3 of the plate was stamped. From this, a fractionated smear was made with a sterile loop.

From punctures (e.g. joint fluids), one drop each from the syringe was dropped on the agar plate and streaked fragmentally. Swab samples were spun down to 1/3 of the agar plate and again a fragmented smear was made. For tonsil samples this was additionally performed on agar plates for the differentiation of streptococci and staphylococci (Oxoid Deutschland GmbH, Columbia-CNA).

The agar plates were then incubated at 37 °C for 48 hours. Afterwards, S. suis suspicious colonies (alpha-hemolyzing colonies) were picked up with a loop. The loop was then smeared fragmentally on a new plate for further incubating the next 24 hours (subcultivation).

S. suis suspect colonies were inoculated in 10 ml of THB and incubated for 12-24 hours in a T405-Cultubes™ (Simport® Scientific Inc., Belœil, Canada) with air exchange. In case of turbidity of the THB medium, the suspension was centrifuged at 2600 g for 5 minutes. The resulting bacterial pellet was stored at -20 °C for DNA.

4.2.9. Detection of the S. suis, serotype 2, strain 10 via multiplex- PCR

To investigate the genomic profile of the bacterial samples coming from blood, CSF or necropsy (see chapter 4.2.8) the material was undergoing a molecular biological examination:

The chromosomal DNA extraction was performed by using DNeasy UltraClean Microbial Kit (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's instructions. Alternatively, in some cases, the DNA preparation was performed by a boiling procedure. Therefore, up to
10 bacterial colonies from the subculture plate were picked up, resuspended in 100µl Sigma (non-pyrogenic) water (Sigma Aldrich, St. Louis, USA) and boiled for 8 minutes in the microwave at 126 W. Afterwards the samples were undergoing the Multiplex PCR according to further studies (Silva et al. 2006) using the TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). The oligonucleotide primer sequences for the respective genes are shown in table 18:

<table>
<thead>
<tr>
<th>PCR assay</th>
<th>Gene GenBank accession number</th>
<th>Primer sequence (5’–3’)</th>
<th>Position in coding sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplex</td>
<td>eap X71881</td>
<td>GCGAGACACGAAAGATTTGA,</td>
<td>2040–2059, 2764–2783</td>
<td>744b</td>
</tr>
<tr>
<td></td>
<td>ept1 AF155504</td>
<td>TGGCCTGATGTGATTTCTCCT,</td>
<td>117–118, 732–127</td>
<td>637</td>
</tr>
<tr>
<td></td>
<td>gdh AF239683</td>
<td>AAGTCCTCCGTTTGGACA,</td>
<td>322–341, 868–566</td>
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<tr>
<td></td>
<td>epe AF118339</td>
<td>TTTGTCGGAAGGTTATTGCC,</td>
<td>330–335, 814–498</td>
<td>498</td>
</tr>
<tr>
<td></td>
<td>ept9H AF1064515</td>
<td>AATTCCTCTGTTGAAATCAGC,</td>
<td>48–67, 407–379</td>
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<tr>
<td></td>
<td>ept9H AF155505</td>
<td>GGGATGTTGCTTGACAGAT,</td>
<td>104–123, 386–303</td>
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<td></td>
<td>sly Z36907</td>
<td>GCCCGCAATACGTAAAGC</td>
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<td></td>
<td>mnp X64450</td>
<td>ATGTGCACCAAGAGGATGG,</td>
<td>3478–3497, 3646–36651</td>
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<td></td>
<td>ora A AF544684</td>
<td>TGATGTTTCAAGGCCTG</td>
<td>1014–1033, 1111–1131</td>
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<tr>
<td></td>
<td>lac U47295</td>
<td>CATAGACAAGCTGCGTGA</td>
<td>634–653, 895</td>
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<tr>
<td></td>
<td>mnp mnp X64450</td>
<td>GAAGATGGTGAGAAAAATGG,</td>
<td>2317–2337, 3646–3665</td>
<td>1148</td>
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<tr>
<td></td>
<td>mnp variant</td>
<td>TAGCCTTCTGTTAGCGGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 18  Genmarker for the differentiation of different S. suis types
Excerpt from Silva et al., 2006; Veterinary Microbiology 115 (2006) 117–127; genes to differentiate S. suis, ST 2, strain 10 are marked with a square.
After amplification of possible gene sequences, the samples were applied with a 100 bp size marker (GeneRuler 100 bp DNA Ladder fragments (ThermoFisher, Waltham, USA)) to a 1.5% agarose gel (Universal-Agarose (peqGOLD); VWR, Radnor, USA) and were separated by gel electrophoresis at 120 volts for 90 minutes. Subsequently, the genes were displayed with a UV transilluminator (Bio-Rad Laboratories, Inc., Hercules, USA) and examined for the presence of the following S. suis serotype 2, strain 10 specific markers: epf (744 bp), gdh (566 bp), cps2 (498 bp), sly (248 bp), mrp (188 bp), arcA (118 bp).

4.2.10. Pathohistological investigations of organs

The tissue samples were examined histopathologically at the University of Veterinary Medicine Hannover, Foundation in the Institute for Pathology in cooperation with Prof. Dr. Andreas Beineke. For this purpose, the samples were fixed in 10% unbuffered formalin for at least 24 hours. Subsequently, the tissue was embedded after mechanical dehydration at 58°C (Shandon Pathcentre, ThermoFisher, Waltham, USA) in a paraffin-Paraplast mixture (VOGEL Histo Comp Paraffin Wachs, Vogel GmbH & Co. KG, Fernwald, Germany) after an ascending alcohol washing series (Tissue-Tek® TEC® 5, Sakura Finetek Europe BV). With the aid of a rotary microtome, 2-3 μm thick serial sections (up to 50 pieces depending on the block thickness) were made. From the sections a haematoxylin-eosin (HE) staining was performed in a stainer (Leica ST 4040, Leica Microsystems GmbH, Wetzlar, Germany). Subsequently, the samples were investigated with the microscope OLYMPUS BX41 (Olympus K.K., Tokyo, Japan) for any signs of infection, in particular for the presence of PMN in the tissues.

4.2.11. In vitro determination of the hygiene status of the measuring system

Since the measuring system cannot be changed during the experiment, it had to be ensured that suitable rinsing processes decontaminate the measuring system in between. The previously described process of sampling and measuring via the measuring system was simulated in the laboratory with S. suis solution. Therefore S. suis serotype 2 strain 10 was smeared on a Columbia agar plate with 6% sheep blood and was incubated for 12-16 hours. After this time a single bacterial colony was picked and added into 10 ml Todd Hewitt Broth (THB) (Becton, Dickinson and Company, Sparks Glencoe, USA). After 17 hours of incubation
the bacterial suspension was mixed with 30 ml of fresh THB. This solution served as a "source of infection" (figure 25). The three-way taps were rotated that way that 5 ml 0.9% NaCl from syringe C could be injected into a syringe at position D (path 1). This moistened the FTC dummies (E). By pulling up and down the syringe A, the bacterial solution was homogenized and the three-way valves were then positioned that way that in addition 1 ml of bacterial fluid could be drawn into syringe D via the CSF-sample-collection path 2. After waiting for 10 minutes, syringe D was disposed of and again via path 1 a volume of 5 ml 0.9% NaCl was injected into a new syringe at position D. After 1, 4 and 7 hours the following procedures were performed:

1) With syringe A the suspension was again homogenized. 2) Via path 1 a volume of 1.8 ml bacterial suspension was aspirated in syringe D; the syringe is discarded. 3) A volume of 1 ml of new S. suis suspension is now aspirated in a new syringe at position D; in parallel 1 ml suspension is also aspirated in syringe B. Both syringes are released and contain comparative samples, which can give information about possible impurities in the measuring path 2. In accordance with chapter 4.1.2, dilution series were applied and smears on blood agar plates were performed. After incubation, the CFU/ml of the two samples were compared. Directly after sampling into syringes B and D, the cleaning path 1 is opened by appropriate rotation of the three-way valves and again 5 ml 0.9% NaCl were injected into a new syringe at position D by syringe C. The syringe (D) is then discarded and replaced with a new one.

The CSF-sample-collection path 2 was built according to the in vivo construction with the same parts. Within this path, a "dead space volume" of 1.3 ml is formed. For safety reasons, 0.5 ml additional volume was added. This results in a total rinse volume of 1.8 ml 0.9% NaCl.
4.2.12. Supplement: Further studies on the oxygen level in CSF post mortem

In 9 pigs that had to be sacrificed in another study, oxygen measurements of the CSF were performed post mortem. The animals were again hybrids from the BHZP breeding stabled in groups in the facilities of the Research Center for Emerging Infections and Zoonoses (RIZ) of the University of Veterinary Medicine Hannover (chapter 4.2.1) but now with a bodyweight of 64 ± 11 kg at the age of 21 to 22 weeks at the time of killing. The animals were anaesthetized by intramuscular injection of a combination of ketamine (20 mg/ kg) and azaperone (2 mg/kg) and about 0.5 liters of blood was taken from each animal. The pigs were subsequently euthanized intravenously with 6 ml T 61 ® (Intervet Deutschland GmbH, Unterschleißheim, Germany). Immediately after detection of death by auscultation of the heart the puncture of the CSF compartment with following oxygen measurement was performed according to chapter 4.2.6.

4.3. Statistical analysis

All experiments for the in vitro part were performed at least three independent times in duplicates (duplicates were averaged) unless indicated otherwise. The in vivo examinations are individual measurements, which are specifically grouped for the statistical evaluation in some cases.
Data were analysed using Excel 2016 (Microsoft) and GraphPad Prism 8.0 (GraphPad Software).

The individual test methods are presented in the respective chapters and figure legends. In case of comparisons the significance is indicated as follows: ns., not significant, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p < 0.0001.
5. Results

5.1. *In vitro* – Investigations on the HIBCPP cell culture

The aim of the *in vitro* studies was to measure the oxygen levels and the pH in case of a *Streptococcus (S.) suis* infection in a model of the blood-cerebrospinal fluid barrier (BCSFB). The model is based on human choroid plexus epithelial cells (HIBCPP) growing on inverted transwell filter. Infection assays with *S. suis* and following human neutrophils (PMN) were performed and the oxygen level as well as the pH were recorded continuously in the lower compartment, mimicking the cerebrospinal fluid (CSF) compartment. The host-pathogen interaction in this compartment is of interest because the immune response here is comparable to the immune response *in vivo* in the early phase of meningitis.

5.1.1. Preparations of *S. suis* stocks

For the infection studies, 3 bacterial batches were prepared (chapter 4.1.1) and the respective colony-forming units (CFU / ml) were determined (chapter 4.1.2). The amount of *S. suis* in the batches varies slightly. The individual batches have the following CFU/ml:
Batch (1): 4.14x10^8 CFU/ml - Batch (2): 2.88x10^8 CFU/ml - Batch (3): 3.21x10^8 CFU/ml

5.1.2. Localisation of bacteria and its influence on the oxygen measurements

According to chapter 4.1.10, differences in the oxygen partial pressures (pO_2) in the measurements between an orbitally shaken (OS) setup and a non-moving (NM) setup may occur. Therefore, as a preliminary experiment to optimize the conditions for the setup, both setups were tested to measure oxygen level in tissue culture medium in the presence or absence of the bacterium *S. suis*. As shown in figure 26 a significant (p<0.01) difference in the pO_2 after 2 hours of incubation was detectable in the presence of the oxygen consumer *S. suis*. While the same oxygen partial pressure of about 130 mmHg was measured in both setups at the beginning of the experiment, a difference of about Δ 27 mmHg was found after an incubation time of 2 hours, although the total amount of oxygen-consuming CFU of bacteria was similar in both setups at time point 2h with or without shaking (figure 26 b).

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Thus, it was the question why similar number of bacteria can lead to different results of oxygen level when the shaking setup was used. The orbital shaker moved the plate with a rotational motion of 15 rounds per minute (rpm), thus it may lead to a different distribution of the bacteria inside the wells and especially on the oxygen sensor spot. To verify this assumption, the CFU were quantified on the sensor spot or on the outer area of the respective well. Confirming this assumption, significantly less bacteria (p<0.01) were found in the orbital shaker-plate directly on the measuring sensor spot (A1) than it was observed in the non-moving model although the total amount of S. suis in the complete well was again nearly the same (figure 27). To avoid that bacteria accumulate on the sensor spot, for all further processes the non-shaking model was used.

Figure 26 Oxygen measurement results in different plate setups
Although both setups had the same oxygen partial pressure at the beginning, there was a significant decrease in the pO₂ on the motionless setup plate after 2 hours of incubation compared to the plate that was continuously orbitally shaken (a). Remarkably, the amount of oxygen consuming bacteria was comparable in both setups at the beginning and after 2 hours (b). Shown are the mean values ± SD of 3 independent experiments (n=3).

Statistics: t-test, n=3; **p <0.01, ns. = not significant
More bacteria were found in the outer circle ($A_2$) of the well in the orbitally shaken plate setup, resulting in significantly fewer bacteria around the oxygen sensor ($A_1$). Nevertheless, the total number of bacteria ($A_1 + A_2$) were nearly the same.

Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: Paired t-test; $n=3$, **$p <0.01$, ns. = not significant

5.1.3. **Determination of the cell number on the filter**

The amount of HIBCPP on the membrane of the filter was calculated by examining 3 excerpts each from microscopic images of 5 filters with TEER ranges between 440 and 520 $\Omega \text{cm}^2$ (figure 28). Two filters had equal TEER and were summarized in the calculation. The values of the counted nuclei per excerpt with a size of $A= 0.0375 \text{ mm}^2$ and the cell number for a total HIBCPP filter are shown in figure 29. The nuclei counting for the sections was performed for 1 filter with a TEER of 440 $\Omega \text{cm}^2$, for 2 filter with 457 $\Omega \text{cm}^2$, for 1 filter with 493 $\Omega \text{cm}^2$ and for 1 filter with 520 $\Omega \text{cm}^2$. The amount of cells in the sections was averaged. Therefore, the mean of cells on a total HIBCPP filter ($A=33.6 \text{ mm}^2$) is calculated as about $2.62 \times 10^5 \pm$ SD $5.9 \times 10^3 \text{ cells/filter}$.
5.1.4. Determination of the barrier integrity and amount of bacteria after transmigration

To monitor the HIBCPP barrier integrity during infection with *S. suis* the TEER and the corresponding dextran flux were measured (chapter 4.1.5). The flux of dextran through nine HIBCPP filter with different TEER values is shown in figure 30. TEER values in the grey-shaded
area correspond to a dextran flow rate of less than 5%, indicating a tight cell barrier. TEER values with more than 500 Ωxcm² show no dextran flux anymore whereas TEER values below 200 Ωxcm² are in the critical range leading to ≥ 5% flux. The dotted lines show the TEER range for the HIBCPP filter (360 – 550 Ωxcm²) that was used in the following experiments.

Figure 30 Flow rate of dextran in HIBCPP filter with corresponding TEER values
As the TEER decreases, the flow rate of dextran increases. A flux of dextran of 100 % corresponds to a missing HIBCPP barrier on the filter TEER values with a corresponding flow rate of dextran < 5% are in the grey marked area. For the infection experiments HIBCPP layer with TEER 360 – 550 Ωxcm² were used.

Figure 31 shows the development of the TEER values in HIBCPP-filter with additional S. suis or S. suis with PMN during the infection experiments (chapter 4.1.7) at the measurements “Start” (measured directly before the infection) and 2 or 6 hours post infection (p. i.), respectively. All filters used in the experiment were in the range mentioned above between 360 - 550 Ωxcm² (fine dotted line). As seen in the figure the TEER values from 3 infection experiments increased slightly during the time of infection for both filter types. Also presented are the CFU/ml at the same measurements. The CFU/ml at the beginning represents the bacterial load in the upper compartment (corresponding to a multiplicity of infection (MOI) of about 15), while the results after 2 hours p.i. represent the CFU / ml in the lower compartment after S. suis transmigration and growth. The results after 6 h p.i. include additionally transmigrated PMN. Differences between the CFU were not significant.
Figure 31. Development of TEER during infection with *S. suis* and PMN transmigration
During an infection a slight increase of the TEER values was observed in HIBCPP-filter with additional *S. suis* and *S. suis* + PMN supplementation. Plotted are the CFU/ml for the respective measurement points and the corresponding TEER. The CFU/ml at “Start” represents the infection dose in the upper compartment, the other CFU represents the number of bacteria in the lower compartments. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: paired t-test, ns. = not significant

5.1.5. Determination of transmigrated PMN

The amount of transmigrated PMN through the HIBCPP barrier was analysed by fluorescence-activated cell scanning (FACS) and revealed an increased transmigration in case of the presence of a stimulus by *S. suis* or by TNFα (20 ng/ml) compared to the transmigration rate of PMN without any stimulus (chapter 4.1.7). The HIBCPP filter were infected with a bacterial amount of about 4.05x10⁶ CFU/ml (corresponding to a MOI ~ 15) in the upper compartment. After 2 hours the remaining bacteria were replaced by a PMN solution containing 1.6x10⁶ PMN. Four hours later (6 h p.i.) an averaged number of bacteria with 5.8x10⁷ CFU/ml ± SD 2.5x10⁷ (figure 31) could be detected in the lower compartment, leading to a transmigration stimulus on the PMN. The experiment was performed three times at different times with changing *S. suis* batches (batch 1-3).

The amount of transmigrated PMN due to the *S. suis* stimulus was 1.39x10⁵/ml ± SD 4.63x10⁴. With the control stimulus by TNFα a lower averaged PMN transmigration of 1.24x10⁵/ml ± SD 1.24x10⁴ was observed. Both values were significantly higher (p<0.05) compared to HIBCPP filters without a stimulus (4.00x10⁴/ml ± SD 2.79x10³) (figure 32).
The amount of transmigrated PMN after 6 h post infection is significantly higher with a corresponding stimulus by *S. suis* or by TNFα (20 ng/ml). Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: one-way ANOVA with follow up Dunnett’s multiple comparison test, n=3, *<0.05; mean ± SD

5.1.6. Oxygen level in the cell culture model

The development of the oxygen partial pressure (pO₂) during the infection experiments (chapter 4.1.7) is shown in figure 33. Initially, the oxygen partial pressure of all samples was in equilibrium with the atmosphere of the cell incubator and indicates a value of about 130 mmHg. While the oxygen level of the medium does not change over time (reflecting the normoxic oxygen level), there was a drop of oxygen in samples with HIBCPP alone or samples with additional *S. suis* and PMN (“infectious filter”) detectable:

After 2 hours of incubation, the oxygen partial pressure in pure HIBCPP filter has dropped to about 70 mmHg and after 6 hours incubation down to about 56 mmHg. The drop of oxygen down to about 22 mmHg in the infectious filter after 2 hours of incubation (marked with (1) in figure 33) was caused alone by the supplemented bacteria, since no PMN were added at this time (for setup reasons). The measurement of the partial pressure 6 hours post infection in these samples however shows a value of about 11 mmHg. At this time, besides the influence of HIBCPP and *S. suis*, the additional effect of the PMN is also reflected. The additional Y-axis shows the measurement results also as calculated percentage (~O₂%) (see explanation in chapter 6.2.1).
Figure 33  **Oxygen partial pressures during the in vitro infection**

The oxygen curve for the HIBCPP alone and HIBCPP with supplemented *S. suis* and PMN are shown for the 0 h ("start"), 2 h p.i. and 6 h p.i. In addition, the stable oxygen level of pure cell culture medium is shown. The decrease of oxygen 2 h p.i. for the "HIBCPP + *S. suis* + PMN" filter reflects only the influence of the bacteria since no PMN were supplemented at this time (1). The values are presented in the units mmHg (torr) and approximate percentage of the total gas pressure. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

To further characterize oxygen depletion under infection, figure 34 shows the measurement results of HIBCPP samples infected with *S. suis*, HIBCPP samples cultured with PMN (transmigration stimulated by TNFα), and samples with HIBCPP alone. After 2 hours of incubation, the samples with *S. suis* showed a drop of oxygen down to about 25 mmHg, the other two samples an approximate drop to 70-80 mmHg. Importantly, at this time no immune cells were added in the "PMN sample" for experimental reasons, so the sample is comparable to the "HIBCPP alone "sample at this time. After 6 hours of incubation however, the *S. suis* sample showed an oxygen partial pressure of approximately 10 - 11 mmHg, whereas the oxygen value for the PMN samples was higher at about 25 mmHg. The drop of oxygen in the HIBCPP alone sample showed values of 56 mmHg at this time.
Figure 34  Influence of *S. suis* or PMN alone on the oxygen content

In addition to the oxygen curve of HIBCPP alone, the influence of further consumption of oxygen by the streptococci or of PMN is shown. The decrease of oxygen 2 h p.i. for the “HIBCPP+PMN+ TNFα” filter reflects only the influence of the HIBCPP since no PMN were supplemented at this time (1). The values are presented in the units mmHg (torr) and approximate percentage of the total gas pressure. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Figure 35 presents the results in another type of plot to give an overview of the oxygen key data in the HIBCPP culture. While the cell culture medium showed constant levels of oxygen after 2 and 6 hours of incubation, there was a significant decrease in oxygen concentration when HIBCPP cells were added. There was no significant difference in the oxygen content between the HIBCPP cells after 2 or 6 hours of incubation. However, the interaction of bacteria or PMN alone with the HIBCPP showed significant oxygen decrease after 6 hours of incubation as mentioned before. The oxygen content in a well with HIBCPP and interacting bacteria and PMN was similar to the results measured with HIBCPP supplemented with *S. suis* alone. PMN alone with HIBCPP also led to a decrease but this was not as strong as the one with *S. suis*. 
While the oxygen content remains constant with pure medium, it drops significantly as soon as HIBCPP cells are added. If further oxygen-consumers are added to the HIBCPP, the oxygen level drop significantly. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: [Column] t-test, ns., not significant, **p ≤ 0.01; ***p ≤ 0.001; mean ± SD

5.1.7. pH level in the cell culture model

At the beginning of the infection experiments (chapter 4.1.7) the pH of all samples was about 7.26. In media alone and in samples with HIBCPP alone a drop of pH occurs down to about 7.0 within the first two hours. In the presence of bacteria, the decrease of the pH was stronger down to about pH 6.8. Importantly, no PMN were added to the sample at this time for setup reasons. While the pH was nearly stable for the next 4 hours in samples with media or HIBCPP alone, a significant decrease in samples with bacteria and PMN was detectable down to pH 6.6 after 6h p.i. (here the additional effect of the PMN is integrated) (figure 36). The corresponding CFU/ml are seen in figure 31.

Additionally, a comparison between HIBCPP alone and samples containing additional S. suis or PMN (transmigration was stimulated by TNFα) is plotted in figure 37. While the pH of HIBCPP and HIBCPP + PMN showed similar values, a significant decrease in samples with additional bacteria was seen after 2 and 6 hours of incubation.
In the experiments a pH of 7.26 was observed at the beginning in all samples. Drops occurred after 2 and 6 h p.i. Whereas the pH curve for medium and pure HIBCPP is nearly the same, the decrease of the pH in the “infection” samples differs and is strong down to pH 6.6 at 6 h p.i. This decrease was significant compared to the pH of pure HIBCPP.

Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: One-way ANOVA with follow up Dunnett’s multiple comparison test, n=3, ***p ≤ 0.001; mean ± SD

In addition to the pH curve of HIBCPP alone, the influence of the streptococci or of PMN (transmigration was stimulated by TNFα) is shown. Whereas the drop in pH for pure HIBCPP and HIBCPP+PMN was nearly the same, a significant decrease of the pH occurred in the HIBCPP filter with S. suis. This decrease was significant compared to the pH of pure HIBCPP.

Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

Statistics: RM one-way ANOVA with follow up Dunnett’s multiple comparison test, n=3, ****p <0.0001; mean ± SD

Figure 38 presents the results in another plot to shall the key data next to each other. A decrease of pH in the media alone was observed within the first two hours of incubation. The
pH was comparable in samples with additional HIBCPP. Within these wells there was no significant pH change during the whole incubation time. A significant decrease was observed if \textit{S. suis} was added to the HIBCPP. The addition of PMN alone was not decreasing the pH.

![Figure 38](image)

\textbf{Figure 38} \textit{Overview of pH level in the cell culture model}

A decrease in the pH was observed during the first two hours of incubation of medium alone. Further decreases occurred in case of additional HIBCPP. The supplementation of \textit{S. suis} alone lead to further drop in pH, whereas the addition of PMN alone do not lead to a decrease in pH. A strong decrease in pH was observed in combination of HIBCPP with \textit{S. suis} and PMN. Nevertheless, this was not significant. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).

\textit{Statistics: two-way ANOVA with Tukey’s multiple comparisons test, ns., not significant, *p ≤ 0.05, **p ≤ 0.01 ; mean ± SD}
5.1.8. Determination of the HIBCPP integrity and oxygen level under 3% oxygen during incubation in a hypoxia glove box

To study the quality of the cell barrier under hypoxia, HIBCPP were exposed to a 3% oxygen atmosphere in the presence or absence of *S. suis* infection.

As initial control experiment, bacterial survival was evaluated under hypoxia compared to normoxia (figure 39). The growth curve of the bacteria was comparable under hypoxia and normoxia until 2 hours incubation, but after 6 hours there was a significant increase in the number of bacteria under hypoxic incubation.

Then the barrier integrity was tested when incubating the HIBCP cells under hypoxia (figure 40). Prior to the start of experiment, the medium was equilibrated to 3% oxygen. As seen in the figures, there was a slight increase in the oxygen level, when the filters were placed in the equilibrated medium. However, the oxygen level dropped to less than 1% in both filter types after about 1 hour, and thus under the minimum of the measurement device. Figure 40 also shows the TEER values during the course of the experiment and indicating high barrier quality under hypoxia in the presence or absence of bacteria.

![Graph showing bacterial growth under hypoxic and normoxic incubation](image)

**Figure 39** Bacterial growth under hypoxic and normoxic incubation

Bacterial growth under hypoxia (3% O₂) and normoxia (~18% O₂) shows comparable values up to a two-hour incubation. After 6 hours incubation, however, there is a significant increase in bacteria under hypoxic incubation. In addition, the oxygen profile of both incubations is shown. Shown are the mean values ± SD of 3 independent experiments (duplicates during the individual experiments were averaged).
5.1.9. Transcript-expression of HIF-1α target gene vegf relative to hypoxic stress

To investigate the relative transcript expression of the HIF-1α target gene vegf relative to the housekeeping gene rsp9 to detect hypoxia stress response in infected and non-infected HIBCCP cells, corresponding measurements were performed (chapter 4.1.12): First, the primer efficiencies for the qPCR for vegf and rsp9 were determined. This primer efficiency results from the plotting of the Ct values related to the corresponding template quantities [ng] as logarithmic diagram and leads to a linear regression. Figure 41 shows an example of the linear regression for the rsp9 primer on pooled uninfected-normoxic samples (chapter 4.1.11). The measurements were repeated 4 times each. Table 19 shows the calculated linear regressions and the associated R² values (quality marker) for all HIBCPP pools. The slope factors (m) were used to calculate the primer efficiencies (E). Results marked with an asterisk were identified as outliers by the Grubbs’ outlier test (α=0.1, GraphPad Prism 8.0).
Figure 41 Linear regression of the Ct values of rsp9 in HIBCPP templates

By the plotting of the log10 amount of cDNA of HIBCPP pool samples (uninfected, 18.5% O₂ incubation) with the corresponding Ct values a linear regression curve can be created. The dotted line represents the amount of template that was used for Ct determination in the individual samples.

<table>
<thead>
<tr>
<th>No infection</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>S. suis</th>
</tr>
</thead>
<tbody>
<tr>
<td>run 1</td>
<td>run 2</td>
<td>run 3</td>
<td>run 4</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th></th>
<th>equations (run 1-4)</th>
<th>R²</th>
<th>m</th>
<th>E [%]</th>
<th>Ø E [%]</th>
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</thead>
<tbody>
<tr>
<td>no infection</td>
<td>Y = -3.676*X + 26.29</td>
<td>0.9867</td>
<td>-3.676</td>
<td>87.08</td>
<td>86.13</td>
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<td></td>
<td>Y = -3.703*X + 26.73</td>
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<td>-3.703</td>
<td>86.23</td>
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<td>-4.629</td>
<td>64.45</td>
<td>66.64</td>
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<td>100.42*</td>
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<td>87.52</td>
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<tr>
<td></td>
<td>Y = -4.989*X + 31.89</td>
<td>0.9715</td>
<td>-4.989</td>
<td>58.65*</td>
<td></td>
</tr>
<tr>
<td>run 6h</td>
<td>Y = -4.159*X + 32.03</td>
<td>0.9877</td>
<td>-4.159</td>
<td>73.96</td>
<td>73.30</td>
</tr>
<tr>
<td></td>
<td>Y = -3.250*X + 30.83</td>
<td>0.9831</td>
<td>-3.250</td>
<td>103.09*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y = -4.110*X + 32.04</td>
<td>0.9775</td>
<td>-4.110</td>
<td>75.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y = -4.300*X + 32.41</td>
<td>0.9929</td>
<td>-4.300</td>
<td>70.83</td>
<td></td>
</tr>
<tr>
<td>run 6h</td>
<td>Y = -3.375*X + 28.61</td>
<td>0.9842</td>
<td>-3.375</td>
<td>97.83</td>
<td>97.80</td>
</tr>
<tr>
<td></td>
<td>Y = -3.464*X + 28.04</td>
<td>0.9882</td>
<td>-3.464</td>
<td>94.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y = -3.294*X + 28.39</td>
<td>0.9894</td>
<td>-3.294</td>
<td>101.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y = -2.306*X + 25.83</td>
<td>0.8926</td>
<td>-2.306</td>
<td>171.43*</td>
<td></td>
</tr>
</tbody>
</table>
By the equations of the linear regressions, the corresponding slopes \((m)\) can be read off. Afterwards the primer efficiencies \((E)\) for vegf and rsp9 can be calculated. Outliers are marked with an asterisk (Grubbs’ outlier test; \(\alpha=0.1\)). The quality of the linear regression is expressed by the factor \(R^2\).

Table 19  **Primer efficiencies of vegf and rsp9 after normoxic and hypoxic incubation**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Equation</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>norm</td>
<td>(Y = -4.036X + 30.82)</td>
<td>0.9735</td>
<td>-4.036</td>
</tr>
<tr>
<td>hypox</td>
<td>(Y = -4.150X + 32.10)</td>
<td>0.9855</td>
<td>-4.150</td>
</tr>
<tr>
<td>norm</td>
<td>(Y = -4.109X + 31.77)</td>
<td>0.9888</td>
<td>-4.109</td>
</tr>
<tr>
<td>hypox</td>
<td>(Y = -4.157X + 31.92)</td>
<td>0.9897</td>
<td>-4.157</td>
</tr>
</tbody>
</table>

**Figure 42  Primer efficiencies of vegf and rsp9 after normoxic and hypoxic incubation of HIBCPP**

Differences between the primer efficiencies in normoxic and hypoxic samples as well as infected or uninfected samples were quantified and need to be considered for calculation of respective transcript expression.

Importantly, it has to be mentioned, that primer efficiency varies comparing infected versus non-infected cells and also normoxic versus hypoxic cells. Thus, primer efficiency needs to be incorporated into the calculation of relative transcript expression.

Finally, under consideration of the respective primer efficiencies, the relative expression of vegf as HIF-1\(\alpha\) specific target gene was analysed relative to the housekeeping transcript rsp9. Figure 43 presents the relative transcription of vegf in non-infected and infected HIBCPP that were incubated under hypoxic conditions in relation to incubations performed under normoxic conditions. Importantly, the relative transcription of vegf is 4-6 times higher in both hypoxic incubated HIBCPP (uninfected and infected) compared to normoxic incubation after an incubation time of 2 hours. This confirms that the cells are responding to the hypoxic conditions by upregulating the HIF-1\(\alpha\) specific target gene vegf, independent of the status of
infection. Differences between infected and uninfected cells were not significant. After an incubation time of 6 hours, however, in both hypoxic HIBCPP incubations (uninfected and infected) no relative transcriptions with a factor above 1 compared to normoxic incubations were detectable any longer.

Figure 43 Relative transcription ratio of vegf in case of hypoxia relative to normoxia
The relative transcription ratio of vegf in HIBCPP after 2 hours of hypoxic incubation (3% O₂) is about 4 – 6 times higher compared to the transcription at normoxic incubation. Differences between infected cells and non-infected cells are not significant. After 6 hours of incubation the relative transcription ratio was < 1. Shown is the mean ± SD of 4 independent experiments.

Statistics: Paired two-tailed t-test; n=4, ns. = not significant

As control experiment, the resulted amplicons of vegf and rsp9 from the qPCR were analysed by their melting points (t=85°C) and their corresponding fragment sizes by gel electrophoresis. A sample is shown in figure 44. The size of the amplicons (regardless of the type of incubation) read with the ladder is 87 bp for testing on rsp9, for testing on vegf it is 344 bp. This reflects the expected cDNA amplicon sizes. The melting curves associated with these amplicons, are the same for both (regardless of the type of incubation) and show a melting temperature of 84.5-85 °C.
5.2. **In vivo – Investigations on the living animal**

The aim of the *in vivo* studies was to measure the oxygen content and pH in the cerebrospinal fluid (CSF) of healthy pigs and pigs suffering from *S. suis* meningitis (intravenously infected). The data were correlated with symptoms of infection. These included the number of bacteria in the blood and CSF, the infiltration of PMN into the CSF as well as the microbiological and histopathological examination of various tissues predisposed to *S. suis* infection.

5.2.1. **Determination of bacterial load in the blood and in the CSF**

Six pigs were infected intravenously with *S. suis* (chapter 4.2.4). These were the individuals H2, H3, H6, H7, H8 and H9 (H = main experiment). The infection dose for the intravenous inoculation of *S. suis* was 3.02x10⁸ CFU/ml (mean, ± SD 4.78x10⁷). Only in the animals H2, H3 and H7 bacteria could be found afterwards in the blood compartment at 13, 16 and 19 hours post infection (p.i.). The animals H6, H8 and H9 however, did not show bacteria in the blood at these time points (figure 45).

In addition, a bacterial invasion into the CSF was also detected in case of the first mentioned animals (H2, H3, H7) as shown in figure 46. Here the CFU is presented at different times. After 16 h p.i. and 19 h p.i. for all three animals (H2, H3, H7) a determination of CFU was possible, whereas after 13 h p.i. a bacterial determination in the CSF was only possible for individual H7 due to technical reasons. No bacteria could be found in the CSF in the animals H6, H8 and H9.
All animals were infected with a comparable dose of *S. suis*. After 13, 16 and 19 hours post infection only in the animals H2, H3 and H7 were bacteria detectable in the blood. In the animals H6, H8 and H9 no *S. suis* was found in the blood at these time points.

Only in animals with *S. suis* load in the blood after 13 h, 16 h and 19 h p.i. (H2, H3, H7) bacteria were also found in the CSF with a tendency of growth. Only in animal H7 it was possible to determine the CFU after 13 h p. i. since enough volume of CSF was available.

Shown are the mean ± SD with the plot of the individual data.
5.2.2. Number of cells and PMN in the CSF and their morphology

5.2.2.1. Determination of cells and PMN in the CSF using FACS

By fluorescence-activated cell sorting (FACS) the number of total cells, as well as the number of singlets and doublets was determined in the CSF (chapter 4.2.8.2). By appropriate gating of the size and granularity, the PMNs were counted separately (figure 47).

![Figure 47](image.png)

**Figure 47** Representative plot of the cells in the CSF counted by FACS
Shown is the amount of singlets in demarcation to clumped cells (FSC-A / FSC-H) (a). Also plotted is the percentage of the total cell amount for gated PMN by forward scatter (FSC-A) and side scatter (SSC-A) (b).

Table 20 shows the individual FACS counted results per time after infection. The table differentiates into control animals (healthy), *S. suis* infected animals with corresponding bacterial findings in the CSF and animals that were infected but showed no bacteria in the CSF. Figure 48 shows the number of PMN/ml in healthy pigs and pigs that got infected but showed no *S. suis* in their CSF (infected) or pigs that showed bacteria in the CSF (infected, CSF *S. suis*). In the calculation outliers were excluded (Rout outlier test (Q=0.5%), GraphPad Prism 8). The data are individually distributed widely. While in the infected animals without *S. suis* in the CSF the measured amount of PMN was lowest, it was slightly higher in the control group (healthy). However, a significant increase can be seen in the animals with corresponding *S. suis* findings in the CSF. Because of the high standard deviations, the increase, however, is not significant.
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animal & 13 hours p.i. & 16 hours p.i. & 19 hours p.i. \\
& all/ml & singlets/ml & PMN/ml & all/ml & singlets/ml & PMN/ml & all/ml & singlets/ml & PMN/ml \\
H1 & control (healthy) & 16400 & 16340 & 150 & 39640 & 38150 & 1040 & 49100 & 48620 & 14050 \\
H4 & 763830 & 734220 & 32390* & 51090 & 50510 & 3720 & 17980 & 17740 & 1220 \\
H5 & 238140 & 233580 & 32390 & 238140 & 233580 & 17130 & 263090 & 257010 & 14370 \\
H10 & 11920 & 11550 & 1590 & 3430 & 3060 & 1430 & 4910 & 4730 & 1370 \\
H11 & 1833120 & 1726920 & 13640 & 24430 & 24340 & 120 & 34750 & 34270 & 547 \\
H12 & 67250 & 65950 & 1240 & 62770 & 60680 & 6750 & 34750 & 34270 & 547 \\
H2 & infected and bacteria in the CSF & 183550 & 181990 & 6000 & 430930 & 421530 & 28630 & 222470 & 218870 & 33020 \\
H3 & 597760 & 588390 & 22270 & 1182460 & 1117750 & 39030 \\
H7 & 121100 & 119740 & 4460 & 24460 & 23810 & 6440 & 19650 & 19230 & 2850 \\
H6 & infected but no bacteria in the CSF & 19030 & 18720 & 3042 & 38500 & 37980 & 6380 & 52850 & 52140 & 6430 \\
H8 & 23920 & 23430 & 3040 & 5430 & 5240 & 1370 & 6210 & 6060 & 1350 \\
H9 & 6270 & 6110 & 640 & 2770 & 2630 & 650 & 2090 & 1930 & 450 \\

Table 20 Individual results of the FACS analysis for the different CSF samples
The table differentiate between the counting of all cells, singlet cells and PMN of all samples. The results are sorted by control animals (healthy), by animals infected with S. suis but without bacterial findings in the CSF and by infected animals with corresponding S. suis finding also in the cerebrospinal fluid. One result could be identified as an outlier (*).

Figure 48 Increase in PMN concentration in the CSF after migration of bacteria
While the PMN concentration in the CSF without S. suis (Infected) remained relatively stable at low level, the PMN concentration increased in samples with additional S. suis findings (Infected (CSF S.suis)). The differences between the groups are not significant however. Shown is the mean ± SD of 3 measurements of each measurement after infection and of each group (except 13 h p.i. Infected (CSF S.suis) n=2).

Statistics: Two-way-ANOVA, ns. = not significant

5.2.2.2. Morphology of cells in the CSF samples
The cells of the CSF samples of H2, H3 and H7 (CSF S.suis) were examined microscopically at different magnifications to check for their morphology after being stained with Diff Quick
staining (chapter 4.2.8.2). In all samples, erythrocytes could be detected. Depending on the sample, different amounts of neutrophilic granulocytes could be observed. In addition, lymphocytes were found regularly in all samples. Figure 49 shows a microscopy images of H3 after 16 h post infection in different magnifications. Shown are a lymphocyte and a neutrophilic granulocyte as well as a typical picture of the erythrocytes in all samples.

![Microscopic investigation of the cells in the CSF of a S. suis positive CSF pig](image)

**Figure 49** Microscopic investigation of the cells in the CSF of a S. suis positive CSF pig

Shown is an example of the CSF investigation from animal H3 (CSF13x8). Lymphocytes, neutrophilic granulocytes as well as a high number of erythrocytes were regularly found in the pictures.

### 5.2.3. Oxygen level in the CSF of pigs

The oxygen levels in the cerebrospinal fluid at 13, 16 and 19 hours after infection were determined (chapter 4.2.6). The results are shown in figure 50 and indicate constant oxygen partial pressures (pO₂) of about 56.2 mmHg - corresponding to about 7 % O₂ - over all time points and experimental groups. Differences between the groups were analysed by a two-way ANOVA but showed no significant differences. The results were grouped for animals that got not infected and animals that got infected with S. suis. Here a further differentiation was made
to animals with subsequent *S. suis* detection in the CSF (infected (CSF* _S.suis_)) or to animals without appropriate findings (infected) (table 21.).

<table>
<thead>
<tr>
<th>13 h p.i.</th>
<th>16 h p.i.</th>
<th>19 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control /healthy</td>
<td>H1, H4, H5, H10, H12</td>
<td>H1, H4, H5, H10, H12</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=5)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>infected</td>
<td>H6, H8, H9</td>
<td>H6, H8, H9</td>
</tr>
<tr>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td><em>S. suis</em> infection</td>
<td>H7</td>
<td>H2, H3, H7</td>
</tr>
<tr>
<td>(n=1)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
</tbody>
</table>

Table 21  Overview of the individual measurements per group and time after infection
For the oxygen level calculation, the individual results were grouped according to the table. Presented is also the number of measurements for each group at each measurement.

The oxygen calculations are corrected for the temperature (rectal temperature) and the prevailing air pressure and pressure of the CSF (p_{air} + p_{CSF}) for each time point. In addition to the oxygen determination in the CSF the corresponding blood oxygenation values (paO_{2}) were recorded. All data are presented in table 22.

![Figure 50: Oxygen level in healthy pigs and pigs that got infected with *S. suis*](image)

The oxygen partial pressure (pO_{2}) in healthy pigs and pigs that got infected with *S. suis* showed nearly a constant value of about 56 mmHg (~7% O_{2}). The plot presents the data separated into healthy pigs, infected pigs with corresponding *S. suis* findings in the CSF (infected (CSF* _S.suis_)) and infected pigs without *S. suis* detection in the CSF. The statistical evaluation (two-way ANOVA) showed no significant differences (ns.) between the groups or inside the groups. Shown is the mean ± SD. The individual grouping per bar is shown in table 21.
Table 22 Oxygen relevant data recorded during the experiments of all animals
Listed are parameters for each individual and measurement that are relevant for the oxygen calculation and interpretation. These include the following parameters: The body temperature directly before the intubation and at the respective measurements, oxygen partial pressure in the CSF and in the blood, the pressure of the CSF and the ambient air pressure. The data are also presented as mean values for each group.

<table>
<thead>
<tr>
<th>animal</th>
<th>infection</th>
<th>pre iso.</th>
<th>13 hours p.i.</th>
<th>16 hours p.i.</th>
<th>24 hours p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T [°C]</td>
<td>pO₂ [mmHg]</td>
<td>pCSF [mmHg]</td>
<td>P&lt;sub&gt;air&lt;/sub&gt; [mbar]</td>
</tr>
<tr>
<td>H1</td>
<td></td>
<td>38,30</td>
<td>81,21</td>
<td>94,50</td>
<td>37,30</td>
</tr>
<tr>
<td>H4</td>
<td></td>
<td>38,50</td>
<td>55,19</td>
<td>89,10</td>
<td>37,30</td>
</tr>
<tr>
<td>H5</td>
<td></td>
<td>38,90</td>
<td>60,22</td>
<td>91,90</td>
<td>37,30</td>
</tr>
<tr>
<td>H10</td>
<td></td>
<td>38,20</td>
<td>67,66</td>
<td>84,20</td>
<td>38,30</td>
</tr>
<tr>
<td>H11</td>
<td></td>
<td>38,40</td>
<td>63,02</td>
<td>92,00</td>
<td>38,80</td>
</tr>
<tr>
<td>H12</td>
<td></td>
<td>38,40</td>
<td>56,21</td>
<td>89,70</td>
<td>38,40</td>
</tr>
<tr>
<td>Ø</td>
<td></td>
<td>38,45</td>
<td>61,92</td>
<td>90,23</td>
<td>37,82</td>
</tr>
<tr>
<td>H2</td>
<td></td>
<td>39,20</td>
<td>-</td>
<td>101,10</td>
<td>39,50</td>
</tr>
<tr>
<td>H3</td>
<td></td>
<td>38,90</td>
<td>-</td>
<td>95,00</td>
<td>37,80</td>
</tr>
<tr>
<td>H7</td>
<td></td>
<td>40,60</td>
<td>58,61</td>
<td>103,00</td>
<td>39,40</td>
</tr>
<tr>
<td>Ø</td>
<td></td>
<td>39,57</td>
<td>58,61</td>
<td>100,23</td>
<td>36,90</td>
</tr>
<tr>
<td>H6</td>
<td></td>
<td>38,90</td>
<td>50,26</td>
<td>105,00</td>
<td>37,70</td>
</tr>
<tr>
<td>H8</td>
<td></td>
<td>37,50</td>
<td>56,42</td>
<td>104,30</td>
<td>37,80</td>
</tr>
<tr>
<td>H9</td>
<td></td>
<td>38,40</td>
<td>59,08</td>
<td>94,90</td>
<td>38,40</td>
</tr>
<tr>
<td>Ø</td>
<td></td>
<td>38,27</td>
<td>55,26</td>
<td>101,40</td>
<td>37,97</td>
</tr>
</tbody>
</table>

5.2.3.1. Influence of blood oxygenation on the oxygen level in the CSF

The influence of the ventilation to the level of oxygen in the CSF (chapter 4.2.7) of one individual pig is shown in figure 51. After a time of physiological blood oxygenation of about p<sub>O2</sub> 100 mmHg and a corresponding “normal” CSF oxygenation (~ 56 mmHg) (chapter 5.2.3) the animal was ventilated with 100% oxygen for 35 minutes. This resulted in an increase of the blood oxygenation to a 3 times higher level than physiological. Surprisingly, a parallel increase of oxygen level in the CSF was not occurring. However, in contrast an immediate drop in the CSF oxygenation within the first 10 minutes after death down to 30 mmHg was detectable, indicating the need to measure oxygen in the CSF in living animals.
5.2.4. Correlations of pO$_2$ in CSF to the amount of bacteria and PMN

To check the correlations of the pO$_2$ and the amount of oxygen consuming agents (PMN / S. suis) all pO$_2$ measurements and the corresponding amounts of the bacteria (data of animals H2, H3, and H7) and the PMN (data of all animals) were collected. The oxygen partial pressure in the CSF was plotted in correlation to these factors (figure 52). The correlations were calculated using the Pearson correlation. For the correlation PMN / pO$_2$, the calculation yielded a value of $r = -0.02714$ with a p-value of 0.8868. Regarding the relationship between S. suis / pO$_2$, the calculation showed a $r$-value of 0.3471 with a p-value of 0.4456. Thus, neither the amount of PMN nor the amount of bacteria in the CSF correlates with the oxygen level.
5.2.5. pH level in the CSF of pigs

In parallel to the oxygen measurements a pH measurement of the CSF was performed (chapter 4.2.6). The pH level in the cerebrospinal fluid at the measurement time points at 13, 16 and 19 hours after infection is shown in figure 53. The data compilation corresponds to the information given in table 21.

The pH values were between 7.32 and 7.44 in all measurements for the control animals and infected animals without bacteria in the CSF (infected). At 16 h and 19 h post infection there was a tendency of decreasing pH down to 6.7 – 6.8 in animals with S. suis findings in the CSF (infected (CSF \text{s.suis})). Nevertheless, the decrease was not significant.

![Figure 53 pH level in healthy pigs and pigs that got infected with S. suis](image)

**Figure 53** pH level in healthy pigs and pigs that got infected with S. suis

The pH level in healthy pigs and pigs that got infected with S. suis but showed no bacteria in the CSF was in constant values between 7.32 - 7.44. This was also the case in infected animals with corresponding S. suis findings in the CSF (infected (CSF \text{s.suis})) after 13 h. p. i. Later there was a drop down to about 6.8.

Shown is the mean ± SD. The amount of measurements per bar is shown in table 21.

**Statistics:** unpaired two-tailed t-test; ns. = not significant

5.2.6. Detection of strain specific markers for the verification of S. suis, ST2, strain 10

According to the Koch’s postulates the infection germ S. suis, serotype 2, strain 10 should be detected in suspicious organs of infected animals - including the cerebrospinal fluid and blood samples (chapter 4.2.8). Therefore, molecular biological investigations were carried out to specify the serotype and strain. Via multiplex PCR and following gel electrophoresis characteristic bands of S. suis, serotype 2, strain 10 (epf, gdh, cps2, sly, mrp, arcA) were
analyzed. Table 23 shows findings with confirmed presence of the inoculation germ in different organs (X). In the infected pigs with corresponding S. suis evasion even into the CSF (H2, H3, H7) the inoculation germ could be detected in all samples with few exceptions. In the infected animals H6 and H9 the inoculation germ was only detectable in the blood, whereas in animal H8 it was only found in the left tarsal joint. In samples from the uninfected control animals H11 and H12 S. suis, serotype (ST) 2 (Cps2) was also detected in tonsils with similar signals but without epf and sly gene expression. Other serotype 2 findings did not occur in the samples.

Table 23  S. suis, ST 2, strain 10 in organ samples of the animals after infection

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Infected (CSF S.suis)</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>X    H4 H5 H10 H11 H12 H2 H3 H7 H6 H8 H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil</td>
<td>!    ! x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carpal+Tarsal joint (left, right)</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>X    x x x x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>X    x x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 24 Overview of samples submitted for histopathological evaluation

5.2.7. Histopathological results

Table 24 lists the organ samples that were histopathologically investigated (X) at the Institute for Pathology, TiHo (Prof. Dr. A. Beineke).
The analysis started with animal H1-H3. However, only non-specific pathological findings were made in most organs. These unspecific findings were:

- Moderate to severe diffuse lymphohistiocytic interstitial pneumonia
- In parts mild to moderate focal villous proliferative synovitis
- Minor multifocal accumulation of neutrophilic granulocytes in the spleen’s red pulp
- Some single detritus-filled crypts in the tonsils

Therefore, for all other animals only the brain was examined. Results with CNS-relevance from the *S. suis* infected animals are listed in table 25.

<table>
<thead>
<tr>
<th>animal</th>
<th>histopathological findings with CNS relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2</td>
<td>None</td>
</tr>
<tr>
<td>H3</td>
<td>moderate multifocal purulent <em>meningitis</em> (cerebellum) low grade lymphoplasmacellular <em>plexus choroiditis</em></td>
</tr>
<tr>
<td>H7</td>
<td>None</td>
</tr>
<tr>
<td>H6</td>
<td>minimal focal lymphohistiocytic <em>plexus choroiditis</em></td>
</tr>
<tr>
<td>H8</td>
<td>None</td>
</tr>
<tr>
<td>H9</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 25 Histopathological findings in the brain of *S. suis* infected animals

Figure 54 shows the microscopical picture of the meningitis finding in animal H3 in the cerebellum in the overview (A) and magnified (B). From the inside to the outside, the *stratum granulosum* (A1), followed by the *stratum gangliosum* (A2) with separate Purkinje cells (A3), as well as the *stratum molendolare* (A4/B4) of the cerebellar cortex can be seen. Overlying parts of the *pia mater* (A5) can be seen with infiltrating neutrophils (B6).

The *plexus choroiditis* from the same animal is shown in figure 55. Inside the choroid plexus a blood vessel (1) with erythrocytes and immune cells as well as the choroid plexus epithelial cells (2) are seen. An infiltration of plasma cells and lymphocytes (3) is occurring.
Figure 54  **Picture of the meningitis from *S. suis* infected animal H3**
Overview (A) and magnified section (B). From the inside to the outside, the *stratum granulosum* (A1), followed by the *stratum gangliosum* (A2) with separate Purkinje cells (A3), as well as the *stratum moleculare* (A4/B4) of the cerebellar cortex can be seen. Overlying parts of the *pia mater* (A5) can be seen with infiltrating neutrophils (B6).

Figure 55  **Picture of the plexus choroiditis from *S. suis* infected animal H3**
Inside the choroid plexus a blood vessel (1) with erythrocytes and immune cells as well as the choroid plexus epithelial cells (2) are seen. An infiltration of plasma cells and lymphocytes (3) is occurring.
5.2.8.  *In vitro* determination of the hygiene status of the measuring system

In order to eliminate possible contaminations of a measurement by the previous measurements during a running 7-hour experiment, the measuring system was rinsed between the measuring steps (chapter 4.2.6). The effectiveness of this cleansing processes has been studied previously in the laboratory (chapter 4.2.11) as control experiment. Figure 56 shows the amount of bacteria that were determined after flowing through the previous rinsed way of the measuring system compared to the amount of bacteria obtained directly from the *S. suis* source. In total, the examination was performed twice with each one “direct source” sample and 2 "FTC samples". In both experiments, there is almost no difference in the CFU/ml between the direct samples and the samples via the "FTC sensors". Due to the *in vitro* demonstrated effectiveness of the rinse cycles between the measurements, the values obtained subsequently in the *in vivo* experiments can be classified as uncontaminated.

![Figure 56](image)

*Figure 56  Effectiveness of the cleaning processes between the measuring points*

To check the effectiveness of the flushing of the FTC measuring system, the *in vivo* setup was mimicked in the laboratory. For this purpose, the CFU / ml were determined from a *S. suis* suspension ("direct") (*in vivo* = CSF) and, in comparison, in each case the CFU / ml which was obtained by pulling the bacteria via the FTC sensor system. The FTC sensor system was duplicated. At the first run (a) the CFU / ml were slightly smaller than in the second run (b). Differences in the CFU/ml between "direct" and "FTC setup" could not be detected. Shown are the means ± SD ("FTC setup") and the total corresponding CFU/ml of the *S. suis* suspensions.
5.2.9. **Supplement: Further study on the decrease of pO₂ post mortem**

In order to further investigate the oxygen decrease in the CSF in a timeframe post mortem, measurements were performed according to the previously described methods on 9 pigs after the death. With this experiment we aimed to see if after a certain period of time after death a similar oxygen level could be detected which we have found under anaesthesia. Depending on how fast the spinal cannula could be inserted into the subarachnoid space, measurements were performed at different times post mortem. The individual oxygen level per minute post mortem are shown in figure 57. In most cases it was shown that the oxygen level of the dead animals was distinctly lower compared to the measured level in living animals (56.2 mmHg / ~ 7% O₂; see figure 50). The decreases of the oxygen level however, showed strong individual fluctuations.

![Oxygen levels in CSF post mortem](image)

**Figure 57 Oxygen levels in CSF post mortem**

Shown are the individual oxygen levels of 9 pigs a few minutes after death. The blue cross marks the value measured on a pig 10 minutes post mortem after isoflurane anaesthesia (chapter 4.2.7). The dotted line represents the level of oxygen previously measured in live animals under isoflurane anaesthesia (chapter 5.2.3).

6. **Discussion**

In the last two centuries, there has been a considerable increase in knowledge in medicine and in the understanding of biological processes. Nevertheless, many pathological processes associated with various diseases are still not understood sufficiently. The increasing threat of global resistance to antibiotics along with the associated difficulties when treating bacterial
diseases (like bacterial meningitis) substantiates the need to deepen research as well as to understand and develop new treatments or preventive strategies. For this purpose, animal experiments are still an essential tool. However, it should be an ethical goal to reduce animal experiments as much as possible and to enhance research on suitable alternative methods (Russell and Burch 1959). The research on cell cultures plays a crucial role here. Currently, however, cell cultures often cannot adequately reproduce the highly complex interactions in a living organism - for example, in the case of infections. Cell-cell interactions cannot be emulated fully, and many other factors often do not match the in vivo situation.

6.1. Terms related to oxygen: Normoxia, physioxia and hypoxia

A prominent example is the factor "oxygen". Living organisms have a highly complex system of oxygenation to compensate for the physical adversities in the solubility of oxygen in liquids (here in the blood) (Sharma and Rawat 2019). While in a classical cell incubator, an oxygen level of about 18-21% - often referred to as “normoxia” - is present, the actual oxygen levels that the cells and organs encounter in vivo are often significantly lower (Carreau et al. 2011). This example already demonstrates the inaccuracies that can arise when using the term “normoxia”. Normoxia does not mean "normal" oxygen level for the cells, instead it refers to the "normal" oxygen level in the room or the cell incubator both of which can even be subject to fluctuations. To describe the "correct" oxygen level for the individual target cells, the term "physioxia" is more appropriate (Carreau et al. 2011).

Increased or decreased oxygen levels are often referred to as “hyperoxia” or “hypoxia”, respectively. However, these terms, universally used, make little sense as “too low” for one cell might be “too high” for another cell. In order to define the term hypoxia more precisely, the following definition can be helpful: “True hypoxia” is the state at the point where oxygen delivery is no longer sufficient to maintain ATP production via oxidative phosphorylation in the single target cell (Gutierrez 1991). No exact biochemical definitions of “hyperoxia” exist, it is therefore sensible to talk about hyperoxia as soon as the oxygen levels are higher than the level in the cell-individual physioxia situation.
6.2. Consideration of physical influences on the oxygen measurements in vitro and in vivo

6.2.1. Adaptation to the air pressure

The level of dissolved oxygen molecules in liquids (e.g. in the blood or cell culture medium) is highly dynamic and is the product of "oxygen consumption" and dissolving molecules from the air. This mechanism follows various laws that are presented in this work (chapter 3.1.2) and that must be taken into account when measuring oxygen values. For example, the air pressure plays a decisive role. Oxygen measurements can therefore differ between individual days (meteorological changes in atmospheric pressure) or between the locations (different attitudes) at which they were performed. Hence, research on the oxygen content in a cell culture can differ significantly between the measuring location "University of Veterinary Medicine Hannover" (65 m) and the university in Los Alamos, USA situated at an altitude of 2231 m. Therefore, the stand-alone indication "21% oxygen" is inaccurate because it is an entity relative to the total air pressure. The actual oxygen levels are described more accurately by the partial pressure of oxygen ($pO_2$) or by absolute concentrations such as mol/l or mg/ml.

However, in regard to the so-called "hypoxia chambers" (chapter 3.1.8) used in laboratories for generating defined atmospheres for cells, mostly the unit "percent" is used.

When measuring oxygen in vitro as well as in vivo, the prevailing air pressures were considered in the calculation. The in vitro measurements were based on the air pressure inside the incubator (or the hypoxia chamber); in vivo measurements also considered the cerebrospinal fluid pressure in addition to the prevailing atmospheric pressure in the room. The sum of both pressures was used as the basis for the oxygen calculation.

The simultaneous plotting of exact oxygen values in a graph as partial pressure and %-indication is only possible for individual values, not for averaged values in most cases due to changed barometric conditions in the individual experiments. This should be considered in the plotting as it was done in this work.
6.2.2. Adaptation to the temperature

In addition to the air pressure, the temperature also has a decisive influence on the oxygen content in the medium. High temperatures lower the solubility of oxygen, while low temperatures increase the solubility of oxygen - and thus the amount of free O\textsubscript{2} molecules in the medium. For a cell culture this means, that physioxia can only be sustained if the temperature is tightly controlled. Fluctuating temperatures, e.g. due to changes of media, freezing processes or other steps between incubator and "laboratory bench" already lead to changes in the oxygen content. For the in vitro part of this work, special care was taken to ensure a continuous temperature of 37 °C by using an accurately prewarmed medium (37°C) and by using heating plates of 37 °C. In theory, cooling of the media leads to an unphysiologically high oxygen concentration for the corresponding cells. It was shown that mitochondria produce more reactive oxygen species (ROS) when exposed to cool media - although the total metabolism in the cells was decreased. Cold cell culture media may therefore lead to oxygen-associated cell stress (Ali et al. 2010).

For the in vivo measurements the temperature management was more difficult. As a basis for the calculation, the body temperature from a rectal measurement right before the CSF sample collection was used. During a time span of 10 minutes the oxygen level of the CSF sample was measured ex vivo. To minimize a temperature drop during this time, the sample was wrapped in aluminum paper. Consequently, the fluorescent dye of the sensor was also protected from light.

6.3. The oxygen supply within the cell culture

Cell vitality is directly linked to the maintenance of ATP production via the respiratory chain (chapter 3.1.5). Cytochrome C oxidase plays a decisive role here. However, it has been shown that this enzyme is still operable at extremely low (near-anoxic) oxygen concentrations (Scandurra and Gnaiger 2010), leading to the hypothesis that the deciding factor for the cell alone is the adequate diffusion of oxygen (Pittman 2011) from the extracellular space. However, this diffusion follows physical laws and quickly reaches its limits. In highly developed animals, the diffusion distance of oxygen is kept as short as possible. Therefore, the distance between the target cell and the blood vessel is very small - usually between 10 – 30 µm (Kety 1951), but not more than 100 µm (Krogh 1919). For cell cultures, this represents a problem.
since the diffusion distance of the oxygen from the air-liquid-interface up to the cell is much larger. The setup of the cell culture, which is described in this work and corresponds to common practice, leads to an oxygen diffusion distance of more than 10 mm (height of 500 µl medium in the column of the filter with a base of 33.6 mm²). Place et al. describe a simplified calculation of the height of the medium column over the cells, in which just enough oxygen can theoretically diffuse so that the oxygen consumption rate of the mitochondria is not undershot. With different oxygen consumption rates (OCR) published by Wagner et al. (Wagner, Venkataraman, and Buettner 2011a) the maximum heights of the media columns were calculated for different cell types and corresponding cell densities. At a high cell density of 100,000 cells / cm² and an OCR in, for example, low metabolic embryonic fibroblasts of mice (MEF) of 40 amol / cell / s, the height of the medium is indicated with max. 12.1 mm. With increasing metabolic activity, the height decreases dramatically. For example, in rat liver cells and a corresponding high OCR of 350 amol / cell / s along with a cell density of 100,000 cells/cm², the maximum medium height is only about 1.5 mm (Place, Domann, and Case 2017).

These theoretical calculations clearly show the problem in cell cultures: If the oxygen consumption rate of the cells exceeds the diffusion capacity of oxygen for any reason, a balance in the oxygen concentration in the medium will fail and will ultimately lead to hypoxic conditions after a correspondingly long cell incubation period directly at the cells. This was also shown in the "hypoxia experiment" described in this work (chapter 4.1.11), in which human choroid plexus epithelial cells (HIBCPP) were exposed to a 3 % oxygen in the hypoxia chamber. After a short time, the oxygen level had dropped below the minimum measurement limit of the SDR sensors of 1%. Due to this very low oxygen partial pressure in the hypoxia chamber, the corresponding diffusion pressure into the medium was correspondingly low. However, since the cells still consume oxygen, this rapid drop in oxygen levels in the cell culture medium was the logical consequence.

In the present work, no oxygen consumption rate of HIBCPP was determined. However, with regard to the secretory properties of the choroid plexus epithelial cells, a higher metabolic activity and thus a tendency towards higher OCR can be assumed. Since the calculations of Place et al. are only based on a maximal cell density of 100,000 cells/cm² it can be assumed, that in the described HIBCPP setup of this work a non-optimal oxygen supply is currently existing because, here, the cell density is more than 7 times higher (2.62x10⁵ cells/filter =
779,762 cells/cm²). Reduction of the medium column over the cells may improve oxygen diffusion if necessary. This consideration has yet to be confirmed in appropriate experiments.

6.4. Methods of the oxygen measurement in vitro and in vivo

In the underlying work, the oxygen concentration was measured by means of an optical detector based on luminescence quenching by oxygen molecules (chapter 3.1.7.1). The advantage of this method compared to other methods is the possibility of continuous measurement, without consuming oxygen molecules by the measurement itself, as it is the case in the alternative polarographic measurement based on (modified) Clark electrodes. The latter is integrated in a number of blood gas analyzers (BGA) such as the epoc device that was used in vivo to determinate the blood oxygenation in the pigs. Clark electrodes are well suited for short-term oxygen measurements. However, if a measurement is to be carried out over a long period of time, the "oxygen consumption" of the Clark electrode can itself falsify the measurement results. This is particularly a problem in closed systems with a small volume. On the contrary, use in large bioreactors or in fluid systems is not a significant problem.

It should be emphasized, however, that the oxygen content measured in vitro was at the bottom of the well. The HIBCPP are suspended 1.01 mm above it on the filter. Thus, the indicated oxygen measurement mostly refers to the "lower compartment" in which the interaction of polymorphonuclear granulocytes (PMN) and Streptococcus (S.) suis takes place in the CSF.

The work demonstrated that moving the experiment setup by an orbital shaker resulted in higher oxygen readings than in static models (chapter 4.1.10). In addition to the assumed better distribution and increased diffusion of oxygen by the movement, it was also possible to reduce the influence of the strongly oxygen-consuming bacteria directly above or on the sensor spot (falsification by a bacterial “microhypoxia” on the sensor). Therefore, all in vitro measurements were performed on a stirred setup.

6.5. Morphological characteristics of the HIBCPP and the function as barrier model for infection research
When working with the cell culture model, structures of overlaying HIBCPP and pleomorphic nuclei (figure 3.4 b) were recognized. This is in accordance with observations from the first description of the cell line and can be explained by the tumorigenic origin of the cells (Ishiwata et al. 2005). Nevertheless, the experiments showed relatively stable or increasing TEER values despite infection with bacteria - in “normoxic” and “hypoxic” conditions. This corroborates the hypothesis that bacterial invasion of the CSF is not only mediated by disruption of the choroid plexus-typical tight junctions, but rather by the transcellular pathway (Wewer et al. 2011). It was also noticeable, that the number of bacteria in the infection model differed after 6 hours of incubation depending on the setup. For example it was observed that the S. suis CFU in wells without PMN interaction was higher than in samples with additional PMN (figure 31). A possible explanation for this could be the initiated PMN defense, such as phagocytosis. This would also be accompanied by the observation that in some PMN - S. suis samples at the FACS count a tendency of a right shift occurred (FSC-A) (figure 19). This indicates an increase in the size of the detected PMN. The interaction of the PMN with the HIBCPP barrier and subsequent successful transmigration could be confirmed in good correlation with previous studies (de Buhr et al. 2016). While there was little passage in the model without a stimulus (4.0x10⁶/ ml), there was a marked increase in transmigration (despite stable levels of TEER) in S. suis triggered passage (1.39x10⁵) or stimulation by TNFα as control (1.24x10⁵/ml).

6.6. Oxygen and pH characteristic of the HIBCPP cell culture

The oxygen characteristic of the cell culture model or the status quo description under "normal" cell incubation atmosphere showed a decrease in the oxygen level in uninfected HIBCPP from the initially approximated 130 mmHg (~18.5% O₂) to values between 70 and 56 mmHg (chapter 5.1.6). The drop in oxygen is based solely on the oxygen consumption of the HIBCPP. In the case of additional bacteria and PMN, a further drop down to 11 mmHg was observed after 6 hours. A comparison of the HIBCPP oxygen curves with either only PMN or only additional S. suis leads to the conclusion that the significant further decrease in oxygen in the simulated infection and immune reaction in the "lower compartment" is mostly due to the additional bacteria and to a lesser extent to the influence of the PMN. To investigate whether this drop in oxygen down to 22 mmHg has an effect on the HIBCPP cells, the cells were exposed to an atmosphere of only 3% oxygen (~ 22 mmHg) for 6 hours.
For this, the cells were incubated in the hypoxia chamber. In addition, a supplementary S. suis infection was partially simulated under this atmosphere. Although the HIBCPP-barrier integrity remained stable (TEER noted to rising and no observed dextran flux), the cells responded to the greatly diminished oxygen environment which, as described above, also quickly fell below the 1% mark. In relation to "normoxic" incubated HIBCPP (~18.5%), an elevated transcription ratio of the HIF-1α target gene vascular endothelial growth factor (VEGF) was noticed, which can be considered an indication for hypoxic stress of the cell. This relative transcription was about 6 times higher compared to normoxic incubation. However, this was observed only with samples exposed to 2 hours of this hypoxic stress. The relative transcription after 6 hours was no longer increased. This may be associated with the brief transient HIF-1α elevation described in the literature, which decreases with prolonged hypoxia and is "replaced" by HIF-2α (Löfstedt et al. 2007). As a transcription factor, HIF also activates other signaling pathways that are relevant for adaptation to hypoxia, e.g. the release of erythropoietin (growth factor for the formation of red blood cells) or several regulators of blood pressure, such as e.g. endothelin (Arbeit 2002). Which target genes in addition to VEGF in the HIBCPP under hypoxia are further up-regulated, was not part of this work but should be further investigated to deepen the characterization of the cells in hypoxic stress.

Besides oxygen, pH was measured during cell culture in the absence or presence of infection. Deviations from the pH optimum can, for example, cause dysfunctional conformational changes in enzymes and thus significantly affect the functionality of the cell or even damage the cell. In order to protect the cells in vitro, a sodium bicarbonate buffer system (NaHCO₃) is usually used in the nutrient medium. As mentioned before, the 1% FCS-medium is a mixture with DMEM / F12 that include NaHCO₃ (1200 mg/l). The buffer system is associated with the CO₂ partial pressure of the incubator atmosphere and is influenced by the chemical balance [CO₂ + 2H₂O ⇄ H₂CO₃ + H₂O ⇄ HCO₃⁻ + H₃O⁺].

Most cells usually get their energy via the respiratory chain if oxygen is available. In the case of an oxygen deficiency, however, energy can also be provided via lactic acid fermentation. This mechanism is energetically less favorable. The thereby increasing lactate concentration is dissipated in vivo via the blood, but in a cell culture, it is reflected in the medium. If the buffer capacities are exceeded, the pH drops. Changes in the pH can therefore also have their cause in the oxygen supply of the cells. Because of the possibility of using the SDR reader to
measure pH values in parallel, these measurements were thus integrated into the infection experiments. Interestingly, a pH drop during the experiment was not only observed in HIBCPP filters, but also in control measurements in pure cell culture medium. If the pH of both samples was initially 7.26, it dropped to 7.00 within 2 hours of incubation in the cell incubator. This decrease may be due to the suboptimal CO$_2$ concentration in the cell incubator (5% CO$_2$) and to the specific 1% FCS-medium mix, although this reflects standard cell incubator conditions. A pH drop down to 6.6 was then observed with additional *S. suis* incubation and PMN transmigration after 6 hours. Similar to the oxygen level, the influence of the bacteria was the decisive factor.

6.7. *Streptococcus suis* infection in the animal experiment

In order to compare the status quo oxygen results (and pH) in the cell culture with the *in vivo* conditions, investigations were performed in pigs infected with *S. suis* (chapter 4.2.4). The pig is a suitable model in this case, because symptoms of a *S. suis* infection at the choroid plexus and the entering of the CSF are similar for both humans and pigs. The oxygen measurement of the cerebrospinal fluid described in this work allowed the determination of oxygen levels after a one-time needle installation in the subarachnoid space (chapter 4.2.6). The three-way valves system prevented the sensors to get in contact with external oxygen. In addition, the pH measurements could be used to rule out any possible air contact of the sensors. This would have resulted in immediate pH increases (see later).

The infection of the pigs was carried out with *S. suis* serotype 2, strain 10. It is described that this strain is highly virulent in piglets (Vecht et al. 1989). The used piglets came partly from different mother sows and were in an exposed age of about 7 - 9 weeks. Experience from previous studies shows that the intravenous infection dose used in this work (effective dose between 2.5 – 3.7x $10^8$) leads to clinical symptoms (Rieckmann et al. 2017).

For animal welfare reasons the piglets were undergoing immediately anaesthesia in case of reaching a score of 25 early, otherwise the long-term isoflurane anaesthesia started regularly 12 hours post infection. Here the animals were stabilized as well as possible to (patho-) physiological vital values that were maintained by medication lege artis. The anaesthesia was performed on cooperation with experts from the Anaesthesia Department of the Small Animal Clinic (TiHo).
Measurements of oxygen and pH were made on a total of 12 pigs, of which 6 were infected. Only in three infected animals bacteria were found in the blood 13 - 19 hours after the infection (H2, H3, H7). In the other three animals, the infectious germ was no longer found at these times. This may be explained by the different immunity of the animals. In this context, reference is made to the conspicuous bacterial findings in two (non-infected) animals on the tonsils. Although it was not detected directly, the infection germ S. suis ST 2 was found. Therefore, it cannot be ruled out that S. suis infections (with serotype 2) occurred previously in the stable of the piglet producer and caused some immunity in some of the experimental animals (active immunity or passive immunity by maternal antibodies). This could explain the elimination of intravenously administered bacteria in three animals.

In the other three animals in which S. suis was found in the blood, there was also a corresponding migration of S. suis into the CSF compartment observed with an increase of CFU/ml between 13 - 19 hours p.i. The assumption that the bacteria have migrated through the blood-cerebrospinal fluid barrier is supported by the histopathological findings of a plexus choroiditis (H3, H6). Only in the case of animal H3, moderate meningitis subsequently occurred in the cerebellum.

It is difficult to say whether this was due to an insufficient number of bacteria and PMN in the CSF or a too short period of infection. An even longer waiting time after the infection could potentially have led to more obvious symptoms. This was, however, not taken into consideration on not-anaesthetized animals for welfare reasons. The alternative of starting the measurements later and keeping the animals under anaesthesia directly after infection (no suffering of the animals by pronounced infection symptoms) would have led to significantly increased anaesthetic risks. This was not justifiable either. In addition, several studies suggest that isoflurane may have negative effects on PMN recruitment (Carbo et al. 2013). By using a setup as presented in this work (anaesthesia – inoculation – wake up – symptoms – anaesthesia), a possible influence was bypassed as well as possible.

The number of bacteria that must be injected intravenously into the pig in order to cause meningitis with corresponding CFU / ml in the CSF is difficult to calculate since, as mentioned above, the CSF volume is not constant but rather a (slow) flow system. However, the
pathohistological findings of a plexus choroiditis in 2/3 animals with *S. suis* detection in the cerebrospinal fluid, as well as the meningitis findings in 1/3 of these animals, indicate an effective infection load by the intravenous injection. A higher infection dose carries the risk of a shock of the animal with possibly peracute dying.

6.8. **Oxygen and pH characteristic of the CSF in healthy and *S. suis* infected pigs**

Analyses of the cerebrospinal fluid of pigs were performed on CSF samples from the cisterna cerebellomedullaris. In order to be as close as possible to the "location" of a meningitis or a potential early immune response to the *S. suis* infection of the CSF via the choroid plexus, this anatomical region at the atlantooccipital joint was chosen.

Interestingly, the oxygen partial pressures in the CSF were relatively stable at about 56.2 mmHg (about 7% O₂) in both, uninfected animals and infected animals. Although bacteria and PMN were detected simultaneously in the CSF, the oxygen level did not decrease in the CSF. A correlation between the number of oxygen-consuming bacteria and PMN in the CSF to the oxygen level was not detectable.

It is conceivable that diffusion effects offset the oxygen consumption by the pathogens and immune cells up to a critical point that was not reached in these experiments. A corresponding increase in the infection dose is, as described above, not readily feasible.

It can be assumed that oxygen efficiently diffuses across the entire surface of the CSF compartment via cell membranes into the CSF. In addition, cerebrospinal fluid is constantly reproduced by the choroid plexus, creating a certain flow. Stromholm *et al*. calculated the CSF production in pigs at about 0.9 ml /min but pointed out possible differences between the pig breeds (Stromholm *et al*. 1994). In humans, the production rate is about 500 - 600 ml per 24 hours (about 0.35 - 0.42 ml / min) (Zettl 2005).

In order to investigate whether diffusion effects prevent oxygen loss in the CSF during a severe bacterial infection with higher bacterial load and subsequent transmigration of immune cells, the experimental setup could be modified by administering streptococci directly into the cerebrospinal fluid under anaesthesia as it was, for example, done in mice with *Streptococcus pneumoniae* (Ribes *et al*. 2017). The expected increase in *S. suis* CFU / ml in the CSF caused by the multiplication of bacteria could then be related to the oxygen content. If the oxygen
content does not decrease, this would prove the diffusion hypothesis even during severe bacterial infections in the CSF.

Parallel to the oxygen measurements, the pH values in the CSF were also determined. Data on the pH value in CSF of pigs are rare in the literature. The presented pH values are approximately 8.1 (Heinritzi 2006; Schillinger 1994). These values appear very high and are in a very atypical range for physiological body fluids. In humans, the pH value is approximately 7.3 (Zettl 2005). In the present work, similar pH values were measured (7.32 - 7.44) (figure 53).

The ability of the CSF to quickly release CO\(_2\) when exposed to air leads to a rapid increase in pH. Measurements of the pH of the CSF must therefore be carried out under strict anaerobic conditions. The high pH values of CSF found in the literature are probably due to this phenomenon. The determination of physiological pH values in the presented setup can - at the same time - be used to check the quality of the oxygen measurement. If significant increases in pH value were observed, it would indicate that the measuring system was leaking. This problem never occurred in this work.

In infected animals, a non-significant tendency for pH drop was seen after 16 and 19 hours post infection. This may be explained by increased bacterial lactate production, infiltrating immune cells or by cerebral effects of infection. However, this phenomenon was observed in animals with and without bacteria. More animals are needed to see if this tendency is biological relevant or not.

6.8.1. Influence of ventilation on the oxygen level in the CSF

In order to investigate any oxygen-affecting effects on the CSF by artificial ventilation during anaesthesia, it was checked if a 35-minutes ventilation with 100% oxygen causes an increase in the oxygen content in the CSF (chapter 4.2.7). While the oxygen partial pressure in the blood increased to 3 times the value of the physiological range, no corresponding increase of the oxygen in the CSF could be detected. This leads to the conclusion that hyper-ventilation with oxygen does not quickly result in an artificial influence on the oxygen content in the CSF. As the blood gases were checked regularly for physiological ranges during the anaesthesia, the ventilation was always adjusted accordingly. Blood gas levels were maintained in physiological ranges in all animals during anaesthesia, therefore the measured oxygen levels in the CSF can
be considered "correct". The physiological arterial pO\textsubscript{2} was maintained in a range between 80 -110 mmHg. Typical arterial oxygen levels in pigs are in this range (Weiskopf et al. 1992).

6.8.2. Decrease of the oxygen levels in the CSF post mortem

A rapid drop in the oxygen levels in the CSF after death of the animals was observed down to 30 mmHg within the first 10 minutes. If a cardiac arrest (absence of a physiological oxygen supply of the cells) is compared to an inadequate ventilation, it can be concluded that the oxygen level in the CSF is not artificially "increasable “(chapter 4.2.7), but insufficient ventilation leads to a decrease of oxygen in the CSF. To prevent that, the blood oxygenation was regularly monitored by blood gas analyses and the ventilation adapted accordingly.

To further investigate the timeframe post mortem, oxygen measurements in CSF were performed on 9 pigs directly after they had died in another experiment. It was found that the post mortem oxygen content declined rapidly, but strong individual differences were observed. Already after 2 minutes post mortem, an oxygen level in the CSF of about 39 mmHg (~ 5% O\textsubscript{2}) was found in one animal. These studies clearly show that it is not possible to measure oxygen levels on dead animals reliably. This confirms the need for the complicated, previously described setup on live animals.

6.9. Comparison of in vitro and in vivo results – conclusion

The present work has shown that there are currently differences between the in vivo situation and the cell culture in regard to oxygen characteristics and pH. These discrepancies may potentially lead to untransferable results from the in vitro research to the in vivo prevailing situation.

If the oxygen levels in vivo remained constant in the CSF at about 56.2 mmHg (~ 7% O\textsubscript{2}) for healthy and S. suis infected animals, the oxygen level in vitro dropped down to 11 mmHg (~ 3% O\textsubscript{2}) in the case of infection with following PMN transmigration. Only in uninfected choroid plexus epithelial cells (HIBCPP) the oxygen values were comparable to the in vivo situation (56 mmHg / ~ 7% O\textsubscript{2} after 6 hours of incubation) (figure 58).
The pH level of the cell culture medium alone when incubated in the incubator at 5% CO₂ showed a drop from 7.26 down to 7.0 within 2 hours and then remained constant at this level for the next 4 hours. The same was observed when HIBCPP were integrated additionally. In the case of an infection with *S. suis* and subsequent PMN transmigration, a further decrease of the pH down to 6.6 occurred.

The pH values *in vivo* were between 7.32 and 7.44 at all measurements for the control animals and infected animals without bacteria in the CSF. These levels were higher than it was observed in the cell culture. At 16 h and 19 h post infection, there was a tendency of decrease in pH down to 6.7–6.8 in animals with *S. suis* found in the CSF. This drop in pH under infection leads to similar values that were found *in vitro* in the infection situation (figure 59). Nonetheless, the conspicuousness of pH levels *in vitro* should be further controlled. The pH drops in the pure cell culture medium down to 7.0 was not expected. Reducing the CO₂ level in the incubator could probably be useful.
If further animal experiments confirm that diffusion effects compensate the oxygen loss in the CSF by bacterial consumers or immigrated immune cells and thus maintain a stable level, this must be taken into account in the cell culture. Since the oxygen loss in vitro is dynamic and depends on various factors, such as cell density, current metabolic rate, behavior of the bacteria, interaction with other cells, etc., the oxygen development cannot be calculated beforehand.

Therefore, a self-regulating system would be needed in which there is a balance between oxygen consumption and diffusing oxygen molecules. For this, it must be ensured that the setup provides the greatest possible diffusion capacity for oxygen. Options would be, for example, to reduce the cell culture medium to minimize the diffusion distance between the air-liquid-interface and the cells maximally. In addition, the use of oxygen-permeable well plates would also be beneficial as already shown in other studies (Zeitouni et al. 2015).

Often, polystyrene-based vessels are used in a cell culture (this also applies to the SDR-OxoDish® OD24 plates used in the present work). Polystyrene has only a low permeability to oxygen (Halldorsson et al. 2015). Alternative plastics based on e.g. polydimethylsiloxanes have a higher diffusion property for oxygen. Thus, oxygen would also diffuse directly from the bottom into the cell medium, bypassing the media column above the cells (Xiao et al. 2015).
Possibly, an increase in the oxygen partial pressure in the incubator (and thus increased diffusion) adapted to the current need for oxygen in the medium (bringing together measurement and O\textsubscript{2} entry, “closed-loop control”) would also enhance the oxygen situation. A similar setup has already been published (Chapple et al. 2016).

In general, "flow systems" in which the cells are supplied with fresh oxygen-equilibrated and pH-adjusted medium on a regular basis and metabolic products are disposed of would best mimic the \textit{in vivo} situation (blood supply). Such systems already exist and are usually addressed as continuous perfusion microfluidic chambers (Rigual-González et al. 2016). However, these are currently not suitable for transmigration experiments, such as oxygen-controlled bioreactor chambers.

Finally, a brief reference is made to the "roller bottle" system, which was developed early in the year 1933 by George Gey (Feller 1950). Here, a bottle filled with only about 20% medium is continuously rotated. Due to gravity, the medium stays down. The adhered cells are therefore only briefly rotated into the medium but are otherwise covered only with a very thin film. As a result, the oxygen diffusion is very high. This principle is only possible with adherent cells. Also, transmigration experiments are of course not feasible here. Nevertheless, this setup illustrates the already partially developed early sensitivity for the sufficient supply of cells \textit{in vitro} with oxygen. In general, however, this sensitivity did not prevail, but has become increasingly important in recent years.
References


Heinritzi, Karl. 2006. *Schweinekrankheiten*. Ulmer UTB.


Lee, Seung-Eun, Hyuk Hyun, Mi-Ryung Park, Youngsok Choi, Yeo-Jin Son, Yun-Gwi Park, Sang-Gi


Tennenbaum, Tobias, David Matalon, Rüdiger Adam, Annette Seibt, Corinna Wewer, Christian


9. Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled

“Measurement of \textit{in vivo} and \textit{in vitro} oxygen levels in the cerebrospinal fluid compartment during infection with \textit{Streptococcus suis}”.

No third party assistance has been used.
I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.
I conducted the project at the following institution(s):

Department of Physiological Chemistry,
University of Veterinary Medicine Hannover

Research Center for Emerging Infections and Zoonoses (RIZ),
University of Veterinary Medicine Hannover

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.
I hereby affirm the above statements to be complete and true to the best of my knowledge.

__________________________________________  _________________________________________

Hannover, 09.09.2019                           Alexander Martens
10. Acknowledgement

First I would like to thank my colleagues from the Institute of Physiological Chemistry and the Research Center for Emerging Infections and Zoonoses (RIZ) at the University of Veterinary Medicine Hannover. Special thanks are to Prof. von Köckritz-Blickwede, whose supervision during the PhD studies, the numerous experiments and the time of development of this work was always very good. Thank you, Maren!

I would also like to thank Prof. Naim as director of the Institute of Physiological Chemistry, that I could be part of his staff. Many thanks also to my two other supervisors, Prof. Valentin-Weigand and Prof. Nau, whose ideas and comments on the project development have always been very constructive. Thank you for your time!

Especially at the beginning of my PhD studies, I was dependent on a lot of help and familiarization with the lab work and especially with the cell culture. This is where my colleague Nicole de Buhr excellently supported me. Thank you, Nicole, for your effort and "forbearance", if it was not quite so fast sometimes ... Thanks also to my colleague Marita Meurer, on whose numerous expertise in the laboratory, I could always fall back spontaneously! Some "big" problems therefore suddenly became "small"...

Collaboration with the colleagues of the Department of Anaesthesia during animal testing was excellent. I really enjoyed the time and learned a lot. Special thanks to Prof. Kästner, Franz, Stephan and Laura!

At this point I would also like to recall the help of the animal keepers of the RIZ, without them the animal experiment phase would have been hard to imagine.

I would also like to thank for support by the colleagues from the Clinic for Swine, Small Ruminants and Forensic Medicine and from the Institute for Food Quality and Food Safety.

Finally, I would also like to thank the Hannover Graduate School for Veterinary Pathobiology, Neuroinfection, and Translational Medicine (HGNI) for their ability to participate in the PhD program "Animal and zoonotic infections". The financial support was provided by the Niedersachsen-Research Network on Neuroinfectiology (N-RENNNT), for which I would also like to thank.
## Score Sheet für die experimentelle Infektion mit *Streptococcus suis*

<table>
<thead>
<tr>
<th>Kriterium</th>
<th>Befund</th>
<th>Score</th>
<th>Befund</th>
<th>Score</th>
<th>Befund</th>
<th>Score</th>
<th>Befund</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Körpertemperatur (rektal gemessen)</td>
<td>&lt; 40°C</td>
<td>0</td>
<td>40,0 – 40,2°C</td>
<td>1</td>
<td>40,3 – 40,5°C</td>
<td>2</td>
<td>&gt; 40,5°C</td>
<td>3</td>
</tr>
<tr>
<td>Nahrungsaufnahme</td>
<td>gut</td>
<td>0</td>
<td>mäßig</td>
<td>1</td>
<td>schlecht</td>
<td>3</td>
<td>schlecht</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(Tier geht sofort zum Trog, Fressdauer mindestens zehn Minuten)</td>
<td></td>
<td>(Tier geht zum Trog, nimmt aber nur geringe Mengen auf, Fressdauer unter 10 Min.)</td>
<td></td>
<td>(Tier zeigt kein Interesse am Futter)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lahmheiten</td>
<td>keine Lahmheit sichtbar</td>
<td>0</td>
<td>ggr. Lahmheit</td>
<td>1</td>
<td>hgr. Lahmheit</td>
<td>3</td>
<td>Festliegen</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>(Entlastung einer Gliedmaße, diese wird jedoch noch aufgesetzt)</td>
<td></td>
<td>(Eine Gliedmaße wird nicht mehr aufgesetzt)</td>
<td></td>
<td>(Tier lässt sich nicht auftreiben), Polyarthritis (hgr. Lahmheit mehrerer Gliedmaßen)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verhalten</td>
<td>frisch</td>
<td>0</td>
<td>matt</td>
<td>1</td>
<td>apathisch</td>
<td>10</td>
<td>zentralnervös gestört (tetanische Krämpfe, Opisthotonus, Ruderbewegungen)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>(Tier ist neugierig, munter, agiert arttypisch)</td>
<td></td>
<td>(Tier wirkt gedämpft, zeigt verringertes Interesse an Umwelt, liegt viel)</td>
<td></td>
<td>(Tier zeigt kein Interesse an Umwelt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Besondere Befunde (Atmung)</td>
<td>costoabdominal</td>
<td>0</td>
<td>abdominal verstärkt</td>
<td>2</td>
<td>Zyanose (Ohren)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Besondere Befunde (Haltung)</td>
<td>arttypisch (Rücken gerade, Kopf in selber Ebene)</td>
<td>0</td>
<td>Kyphose</td>
<td>2</td>
<td>Tremor (Zittern über mehrere Körperteile)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbruchkriterien:** Tiere, die einen Score von 25 oder darüber erreichen, werden umgehend narkotisiert.