Aus der

Universitätsklinik

für Allgemeine, Viszeral- und Transplantationschirurgie Tübingen

Hyperthermic Pressurized IntraPeritoneal Aerosol Chemotherapy (hPIPAC) – A pharmacological study ex vivo

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

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2023

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Tag der Disputation:	26.01.2023

Meiner Mama und Omi

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abs	Absorption
ADP	Adenosine diphosphate
BSA	Body skin area
°C	Degree Celsius
CAWS	Closed aerosol waste system
CI	Confidence interval
Cm	Centimeter
CO_2	Carbon dioxide
CRS	Cytoreductive surgery
CT	Chamber temperature
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
ELP	Elastin like peptide
ePIPAC	Electrostatic precipitation pressurized intraperitoneal aerosol chemotherapy
ex	Extinction
FPH	Fisher & Paykel Healthcare
g	Gram
GLP	Good laboratory practice
HEPA	High-efficiency particulate air/arrestance
hIBUB	Hyperthermic inverted bovine urinary bladder
HIPEC	Hyperthermic intraperitoneal chemotherapy
hPIPAC	Hyperthermic pressurized intraperitoneal aerosol chemotherapy
HPLC	High performance liquid chromatography
IBUB	Inverted bovine urinary bladder
IL	Interleukin
IP	Intraperitoneal
l	Liter
LLoQ	Lower lever of quantification
LPS	Lipopolysaccharide

m^2	Square meters
m^3	Cubic meter
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mmHg	Millimeter of mercury
mol	Molar mass
NaCl	Sodium chloride
NCPP	National Center for Pleura and Peritoneum
ng	Nanogram
nm	Nanometers
OT	Object temperature
pH	Potential of hydrogen
PDT	Photodynamic therapy
PIPAC	Pressurized intraperitoneal aerosol chemotherapy
PTT	Photothermal therapy
RFA	Radiofrequency ablation
RT	Room temperature
s	Second
TNF-α	Tumor necrosis factor alpha
USA	United States of America
vs.	Versus
µm	Micrometer

1 Introduction

Peritoneal metastasis defines intraperitoneal dissemination of metastases that do not originate from peritoneal tissue. An estimate of 20 000 new cases of peritoneal metastasis are diagnosed in Germany every year (Piso & Arnold, 2011). The most common primary tumors include gastric, ovarian, colonic, rectal, and appendiceal cancers.

Peritoneal metastasis of gynecological and gastrointestinal origin remains one of the most significant oncologic challenges. Despite significant recent advances in cancer treatment, peritoneal metastasis will be the ultimate cause of death in nearly 100 % of cancer patients. Depending on the primary tumor, median survival is measured in months (Elias et al., 2001; Jayne et al., 2002; Lemmens et al., 2011; Marz & Piso, 2015; Sadeghi et al., 2000).

Therapy of peritoneal metastasis is usually palliative, intending to prolong life and preserve the quality of life. Systemic chemotherapy is the mainstay of treatment, but peritoneal metastasis is relatively chemoresistant (Franko et al., 2016). Long-term survival in peritoneal patients is rarely achieved even with the most frequently used systemic therapies (Dahdaleh & Turaga, 2018).

Morbidity of peritoneal metastasis remains high, and patients often suffer from symptoms and complications, influencing their life quality a negative way. Patient management includes abatement of symptoms and psycho-oncological assistance, addressing emotional and existential issues (Lambert & Hendrix, 2018).

Chemotherapy is the core of palliative cancer therapy. However, the clinical use of chemotherapeutic drugs possesses a limited therapeutic index in which leads to unacceptable toxicity, a lack of tumor selectivity, or multiple drug resistance. Strategies delivering these drugs directly to the location of the tumor should improve the therapeutic index and provide additional benefits for the patient (Moktan et al., 2012).

When cancer spreading is limited to the peritoneal cavity, a local dose intensification using intraperitoneal drug delivery might improve the cytotoxic effect of chemotherapy. Enhanced cytotoxicity is based on the theoretical potential for increased exposure of the tumor to antineoplastic agents during intraperitoneal delivery (Markman, 2003). This combined approach finds little attention in Europe and the USA (Markman, 2015), but is widely used in Asia (Yonemura et al., 2019).

Intraperitoneal chemotherapy was described for the first time almost 70 years ago (Economou et al., 1958), but its effect on the macroscopic peritoneal disease was limited. Over the last 30 years, a new procedure, combining complete cytoreductive surgery (CRS) for eliminating macroscopic disease and hyperthermic intraperitoneal chemotherapy (HIPEC) for treating residual microscopic disease, has been applicated increasingly.

Significant pharmacological determinants of intraperitoneal chemotherapy are choice of drug, drug dosage, solution volume, carrier solution, intra-abdominal pressure, temperature, duration, mode of administration, the extent of peritonectomy, and interindividual variability (de Bree et al., 2017). Commonly used drugs include mitomycin C, cisplatin, carboplatin, oxaliplatin, irinotecan, 5-fluorouracil, gemcitabine, paclitaxel, docetaxel, doxorubicin, pemetrexed, and melphalan (de Bree et al., 2017; Mistry et al., 2016).

Based on statistics showing long-term survivors, CRS and HIPEC might have a curative potency in highly selected patients (Chia et al., 2016). However, the level of evidence for CRS and HIPEC is still relatively limited (Ceelen, 2019), and the significant rate of complications remains a hurdle to the wide-spread application (Sugarbaker, 2012). Consequently, there is a need for novel therapeutic approaches to be developed for the majority of peritoneal metastasis patients who cannot participate in current CRS and HIPEC regimens as they are unsuitable for these treatments due to their age, physical condition, or extent of metastasis in the peritoneal cavity (Sleeman, 2017).

1.1 Role of hyperthermia for enhancing target effect of intraperitoneal chemotherapy

The combination of chemotherapy with hyperthermia is assumed to be key to the cytotoxic efficacy of HIPEC (Quenet et al., 2018). Previous work suggests that adding hyperthermia could enhance both the pharmacological and biological effects of intraperitoneal chemotherapy in the target tissue. According to the Einstein–Stokes equation, the transmembrane transport of small molecules is driven by diffusion. Diffusion is proportional to temperature (Reeks, 2011), which is why increased drug diffusion can be expected with higher temperatures. Larger molecules, such as most chemotherapeutic drugs, cannot be transported by diffusion because they require fluid transport (so-called convection). The primary determinant of convective transport, hydraulic conductivity, is also increased at a higher temperature (Carlier et al., 2017). The thermal enhancement of the drugs' activity and penetration depth is often already observed at temperatures above 39 – 40 °C (de Bree et al., 2017).

For example, in the swine model, adding hyperthermia to elevated intraabdominal pressure further elevated the tissue concentration of cisplatin (Facy et al., 2012). Recently, hyperthermia was shown to delay the repair of DNA damage caused by cisplatin or doxorubicin by blocking histone poly-ADP-ribosylation efficiently, producing a comparable delay in DNA repair, induction of double-strand breaks, and cell cytotoxicity after chemotherapy (Schaaf et al., 2016).

However, in a rodent model, mild hyperthermic perfusion with cisplatin (40 °C for 90 min) did not improve drug uptake into peritoneal nodes (Zeamari et al., 2003). In another rodent model for ovarian cancer, hyperthermic chemoperfusion (42 °C for 60 min) was ineffective for enhancing cisplatin concentration in tumor nodes (Facy et al., 2011). There is no comparative study showing that HIPEC is superior to normothermic intraperitoneal chemotherapy (de Bree et al., 2017). Thus, there is still controversy about in how far hyperthermia can enhance the target tissue effect of intraperitoneal drug delivery, and more detailed knowledge is needed about how hyperthermia exerts its effects on chemotherapy.

1.2 Optimizing intraperitoneal drug delivery

There are two limiting pharmacokinetic problems which effect the intraperitoneal chemotherapy: first, the low drug tumor penetration and second, the incomplete irrigation of serosal surfaces by the drug-containing solution (Dedrick & Flessner, 1997; Flessner, 2016). Furthermore, intraperitoneal chemotherapy is impeded by dose-limiting local toxicity (Markman, 2015). Therefore, there is a need for next-generation intraperitoneal drug delivery systems for intraperitoneal chemotherapy that maximize local efficacy while limiting systemic side effects (Dakwar et al., 2017).

1.3 Pressurized intraperitoneal aerosol chemotherapy (PIPAC)

A new method to treat peritoneal metastasis is pressurized intraperitoneal aerosol chemotherapy (PIPAC) (Reymond et al., 2000). PIPAC is the sum of two procedures: (1) a conventional staging laparoscopy and (2) the application of a therapeutic aerosol into the abdomen. A dedicated laparoscopy is not always needed since most patients with peritoneal metastasis have at least one staging laparoscopy, and the mean number of PIPAC/patient is 2.3 (Registry, since 2017).

“The rationale behind PIPAC is:

- *optimizing homogeneity of drug distribution by applying an aerosol rather than a liquid solution;*
- *applying increased intraperitoneal hydrostatic pressure to counteract elevated intratumoral interstitial fluid pressure;*
- *limiting blood outflow during drug application;*
- *steering environmental parameters (temperature, pH, electrostatic charge, etc.) in the peritoneal cavity for best tissue target effect.*

In addition, PIPAC allows repeated application and objective assessment of tumor response by comparing biopsies between chemotherapy cycles. Although incompletely understood, the reasons that allow PIPAC to overcome established chemoresistance are probably linked to local dose intensification. All pharmacological data published so far show a superior therapeutic ratio (tissue

concentration/dose applied) of PIPAC vs. systemic administration, of PIPAC vs. intraperitoneal liquid chemotherapy, of PIPAC vs. Hyperthermic Intraperitoneal Chemotherapy (HIPEC) or PIPAC vs. laparoscopic HIPEC." (Nadiradze et al., 2019)

Known limitations of pharmacological chemotherapy could be solved by a special drug delivery system called pressurized intraperitoneal aerosol chemotherapy (PIPAC). PIPAC might play an important role in improving the efficacy of intraperitoneal chemotherapy and reducing its toxicity, as shown in preclinical experimental studies, patient cohorts in several indications with different drugs and Phase-I and Phase-II studies (Alyami et al., 2019).

PIPAC is not an explicit therapy, it is rather seen as a general system for (intraperitoneal) drug delivery that is able to aerosolize an extensive variety of substances for a diversity of diseases and indications. The real but "nonspecific" effect of PIPAC in various tumors with arbitrary drug choice and formulations appears puzzling at first. This is difficult to comprehend considering that there is a dose reduction of chemotherapy. However, synthesis of all data available (at this point in time including over 80 preclinical and clinical studies) strongly suggests that PIPAC increased efficacy is induced to the highly effective mode of distribution of a drug into tumor nodes rather than determining to a particular medicine. A possible reason for this enhanced target effect is that the application of an artificial hydrostatic pressure to the abdominal cavity (Esquis et al., 2006; Jacquet et al., 1996) helps overcoming elevated intratumoral interstitial fluid pressure (Heldin et al., 2004). Therefore, PIPAC dramatically increases tissue drug uptake, as shown both in preclinical and clinical studies (Solass et al., 2014; Tempfer et al., 2018).

The first PIPAC application in a human patient was done on 05.11.2011 in Bielefeld by Prof. M. A. Reymond (Solass et al., 2014). Since then, over 12 500 PIPAC applications have been performed worldwide. The conclusions of a recent systematic review of a total of 106 articles on PIPAC, with 45 clinical studies about 1810 PIPAC procedures on 838 patients, were that *“PIPAC has been*

shown to be feasible and safe. Data on objective response and quality of life were encouraging. PIPAC can be considered as a treatment option for refractory, isolated peritoneal metastasis of various origins." (Alyami et al., 2019)

Specifically,

- The frequent utilization of PIPAC was practicable in 64 % of patients.
- There were few intraoperative (3 %) and postoperative (3 %) surgical complications.
- In 12 – 15 % of procedures there have been adverse events (Common Terminology Criteria for Adverse Events greater than grade 2). They commonly contained bowel obstruction, bleeding, and abdominal pain.
- The repeated application of PIPAC did not affect the quality of life negatively.
- The objective clinical response after PIPAC was:
    - Ovarian cancer; 62 – 88 %,
    - Gastric cancer: 50 – 91 %,
    - Colorectal cancer: 71 – 86 %,
    - Malignant peritoneal mesothelioma: 67 – 75 %.
- After all the median overall survival was
    - Ovarian cancer: 11 – 14 months,
    - Gastric cancer: 8 – 15 months,
    - Colorectal cancer: 16 months,
    - Malignant peritoneal mesothelioma: 27 months. (Alyami et al., 2019)

Further studies will be needed to validate the use of PIPAC in further indications.

2 Introducing hyperthermic PIPAC (hPIPAC)

PIPAC, in contrast to HIPEC, has been performed at body temperature under normothermic conditions up to now. Since hyperthermic conditions have been claimed to be critical to HIPEC's efficacy, there is a strong demand among HIPEC surgeons to enhance PIPAC technology with hyperthermic features, hoping to further increase the antitumoral effect.

The first preclinical study on hyperthermic PIPAC (hPIPAC) was published at the end of 2016 by a Korean group (Jung do et al., 2016). The corresponding system consisted of a laparoscopic nebulizer and an extracorporeal heater generating hyperthermia by a continuous flow of heated, dry CO_2. They then performed a laparoscopy gastrectomy on five healthy pigs and administered cisplatin 25 mg under mild hyperthermia conditions (38.8 – 40.2 °C). All animals survived the procedure. At the autopsy seven days after the procedure, no tissue lesion was observed at microscopy in the stomach, peritoneum, and jejunum. No pharmacological data on tissue drug concentration and homogeneity of drug distribution were provided.

A few months later, a group from China published a preclinical study using nude mice investigating the effect of warm humidified CO_2 (43 °C, 95 % relative humidity) in comparison to dry cold (21 °C, < 1 % relative humidity) CO_2 after intraperitoneal injection of human colon cancer cells (SW116) (Peng et al., 2017). They documented a protective effect of warm, humidified CO_2 against the peritoneal dissemination of tumor cells. However, no therapeutic aerosol was applied in this particular study. Another group documented a protective effect of warm-humidified CO_2 against tumor cell adhesion and growth onto the peritoneum (Carpinteri et al., 2015).

Recently, our research group at the National Center for Pleura and Peritoneum (NCPP) in Tübingen successfully set up a relatively simple technology for generating and maintaining hyperthermia during PIPAC (Bachmann et al., 2021),

which is a real advance in the field. Therapeutic hyperthermia (target tissue temperature 41 – 43 °C) could be established and maintained over 30 minutes. In the first phase (insufflation phase, open system), tissue hyperthermia was created by insufflating warm-humid CO_2 using a modified industry-standard device. In a second phase (aerosolization phase, closed system), chemotherapeutic drugs were heated up and aerosolized using an angioinjector. In a third phase (application phase, closed system), hyperthermia was maintained within the therapeutic range using an endoscopic infrared heating device. In a fourth phase, the toxic aerosol was discarded using a closed aerosol waste system (CAWS). The principle of hPIPAC is illustrated in Figure 1.

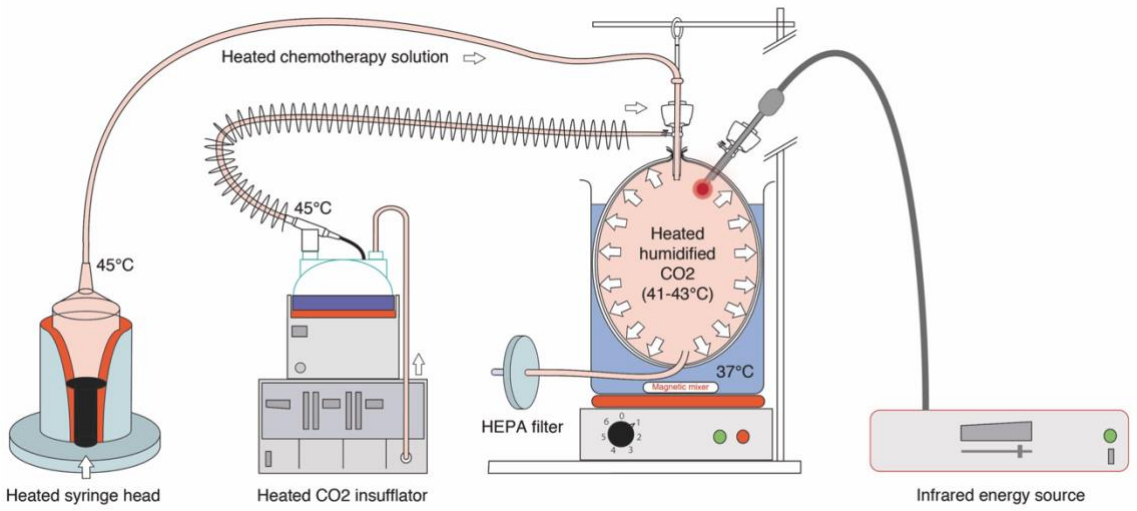

Figure 1: "*Technology proposed for generating hyperthermic pressurized intraperitoneal aerosol chemotherapy (hPIPAC).* *The system consists of the following components, connected sequentially: an angio-injector equipped with a heating cuff; a CO_2-insufflator delivering dry CO_2 at a temperature of 33 °C; a device humidifying and warming up CO_2 to an output temperature of 45 °C and an endoscopic infrared sapphire coagulator inserted into the lumen of the hIBUB model."* Reproduced with permission from Bachmann et al. (2021).

3 Objectives

To our knowledge, no data are available comparing the pharmacological effects of PIPAC under hyperthermic (41 – 43 °C) vs. normothermic (37 °C) conditions. In the present study, we evaluate the pharmacokinetic impact of hPIPAC in the IBUB model (Bachmann et al., 2021). The hyperthermic IBUB is an established ex-vivo model in which the physicochemical properties of a therapeutic aerosol and its effect on the mesothelial tissue can be easily evaluated.

Specifically, we made the following hypothesis:

- hPIPAC does not cause microscopic tissue damage,
- hPIPAC increases tissue concentration of cisplatin and doxorubicin compared to PIPAC,
- hPIPAC increases the depth of tissue penetration of doxorubicin compared to PIPAC.

4 Material and methods

4.1 Study design

This is an experimental study using the established ex-vivo bovine urinary bladder model. In this study the effects of the normothermic and hyperthermic PIPAC were compared.

As recommended by the University of Tübingen and following the good scientific practice standards of the German Research Foundation, all research findings were documented. Our research group (AG PIPAC) uploaded all experimental results to the LabGuru platform.

4.2 Ethical and regulatory background

This study did not include any human patients or tissues. Therefore, there was no need for a permit of the ethics committee of the University of Tübingen. For the experiments fresh biological tissues of animals from the slaughterhouse were used. For conducting this experimental study, there was no requisition of an Animal Protection Committee because no living animals were sacrificed.

4.3 Pressurized intraperitoneal aerosol chemotherapy (PIPAC)

Pressurized intraperitoneal aerosol chemotherapy (PIPAC) is a minimally invasive surgical drug delivery system aimed at optimizing the distribution of (chemo-) therapeutic agents within the abdominal cavity and especially the visceral- and parietal peritoneum. The principle of PIPAC is illustrated in Figure 2.

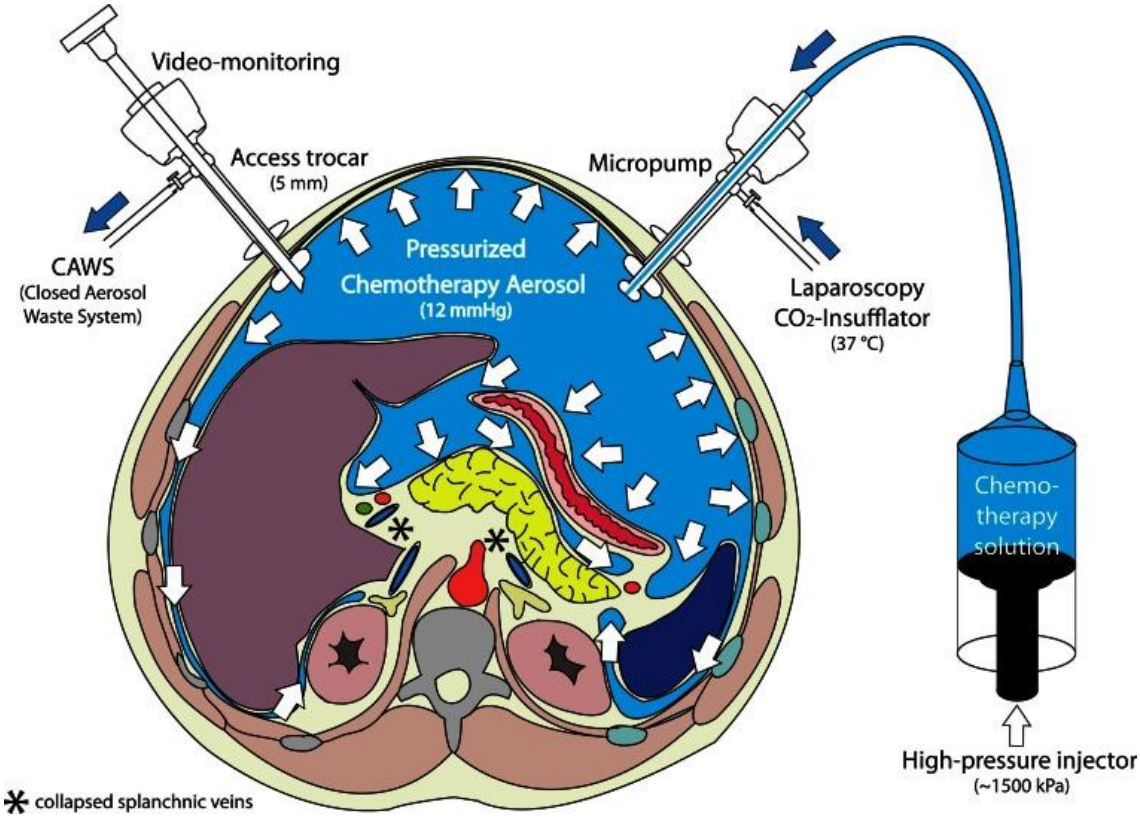

Figure 2: Principle of pressurized intraperitoneal aerosol chemotherapy (PIPAC). Reproduced with permission from Solass et al. (2014).

The procedure is performed in an operating room equipped with an advanced ventilation and filtering system. It is remote-controlled and includes the following steps:

- A normothermic CO_2-pneumoperitoneum is established at intraperitoneal pressure of 12 – 15 mmHg, ascites is removed, and a staging laparoscopy including tumor biopsies is performed.
- Capnopen® is inserted into the abdomen through an industry-standard trocar (e.g., Kii®, Applied Medical). The tightness of the abdomen is controlled.
- The therapeutic aerosol is generated by remote activation of an industry-standard angioinjector with an upstream pressure of 12 – 20 bar and a liquid flow of 0.5 – 1.0 ml/s. There is no gas flow, so that intraperitoneal pressure remains constant at 12 – 15 mmHg.
- This steady state is maintained for 30 min (application phase).

- The therapeutic aerosol is discarded over a single-use, closed aerosol waste system (CAWS) consisting of tubing with two sequential filters connected to the anesthesia gas scavenger system of the hospital.
- The PIPAC procedure is terminated.

4.4 Ex-vivo, inverted bovine urinary bladder (IBUB) model

In our research laboratory the inverted bovine urinary bladder (IBUB) is established and has been described previously (Sautkin et al., 2019; Schnelle et al., 2017). The IBUB model is particularly suitable for the development of optimizing peritoneum targeting drug delivery systems. At the beginning of this laparoscopic procedure, the bovine urinary bladder is filled with 3 – 5 liters of CO_2, equivalent to the human abdominal cavity volume. In the living bovine the bladder lies intraperitoneally and is almost completely covered with peritoneum. By inverting the bladder, the inside lumen is then covered by a homogeneous peritoneal layer with the urothelium on the outside. This experimental setup affords the opportunity to evaluate the drug penetration and depth into the serosal tissue.

For these experiments, fresh bovine urinary bladders, obtained from the slaughterhouse, were transported to our laboratory at a temperature of 4 – 8 °C. Upon receipt, the organs were thoroughly cleaned inside and outside with water. Afterwards, a 2 cm incision was made in the bladder neck, and the organ was carefully inverted through said incision. After that, a 12 mm balloon trocar (Kii®, Applied Medical, Düsseldorf, Germany) was inserted in the open bladder neck, fixed tightly with a Mersilene® purse-string suture, and secured by inflating the trocar's balloon.

4.4.1 Hyperthermic IBUB model

The IBUB model was modified previously to allow experiments under therapeutic hyperthermia conditions (Bachmann et al., 2021). CO_2 was insufflated at a pressure of 15 mmHg via an industry-standard, CE-certified laparoscopic insufflator equipped with an integrated heating element (Endoflator, serial number: 26430520, Karl Storz GmbH, Tuttlingen, Germany). A prototype derived

from an industry-standard surgical humidification CO_2 system (Humigard®, Fisher & Paykel Healthcare, Auckland, New Zealand) was developed to provide warm, humidified CO_2 to the bladder at a temperature over 41 °C, sufficient to generate intraluminal hyperthermia with a usual CO_2 flow. Furthermore, for keeping the intraluminal hyperthermic conditions (41 – 43 °C) during the steady state (exposition time of 30 min), additional heat has provided to the system using an endoscopic infrared heating device (Licht Koagulator, LC250 NK-Optik, Munich, Germany) (Bachmann et al., 2021).

4.4.2 Heat loss simulation

In human patients, blood at body core temperature (37 °C) entering the heated peritoneal volume results in heat exchange (local temperature loss and systemic temperature gain) (Kok et al., 2017). In order to take this heat exchange into account and simulate clinical conditions properly, the IBUB was immersed into a water bath (Thermo-Temp, MGW Lauda, Lauda-Königshofen, Germany) maintaining at a constant temperature of 37 °C.

4.4.3 Temperature measurements

Figure 3 shows the locations of temperature measurements.

(1) the temperature of CO_2 was measured within the IBUB (Folding infrared thermometer, Testo 104-IR, Testo, Reutlingen, Germany).

(2) the target tissue temperature was measured at the external surface of the IBUB just above the surface of the water bath (Infrarot Thermometer, ScanTemp385, Dostmann electronic GmbH, Mannheim, Germany).

(3) the temperature of the water bath (37 °C) was monitored continuously (Thermohygrometer, Amarell, Kreuzwertheim, Germany).

All thermometers were gauged before measurements.

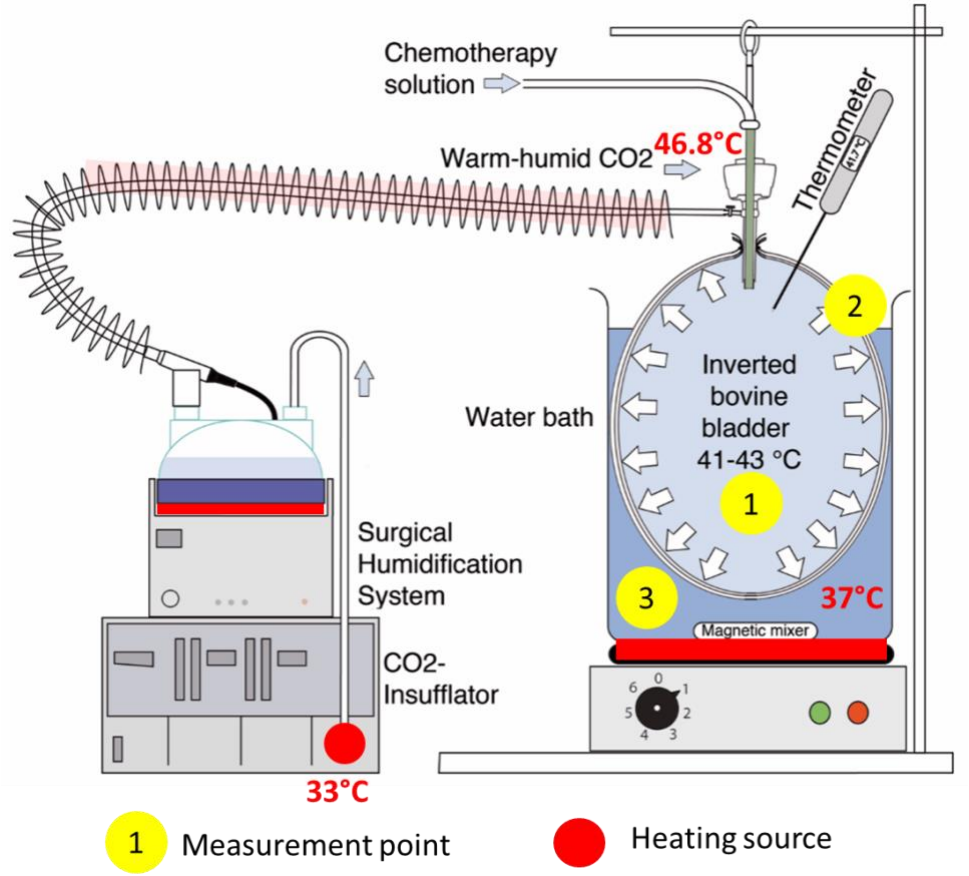

Figure 3: Locations of temperature measurement points at the experimental setup of hyperthermic pressurized intraperitoneal aerosol chemotherapy (hPIPAC) experiments in the inverted bovine urinary bladder (IBUB) model. The IBUB is immersed into a water bath maintained at a constant temperature of 37 °C. Intraluminal hyperthermia is provided by a device generating heated, humid CO_2 (modified prototype from Humigard, Fisher & Paykel Healthcare, Auckland, New Zealand). Legend: Red: heating source. Yellow: temperature measurement point. 1: Exit of the CO_2-insufflator (Endoflator, Karl Storz GmbH, Tuttlingen, Germany); 2: Laser thermometry at the external aspect of the IBUB, directly above the water surface; 3: Temperature of the water bath.

4.4.5 Environmental characteristics

The experiments were performed at room temperature (between 22 and 24 °C), and air humidity was monitored (between 30 and 50 %).

4.4.6 Homogeneity of temperature distribution

Before assessing pharmacological parameters, homogeneity of temperature within the IBUB was tested and the model optimized until this temperature was homogeneous.

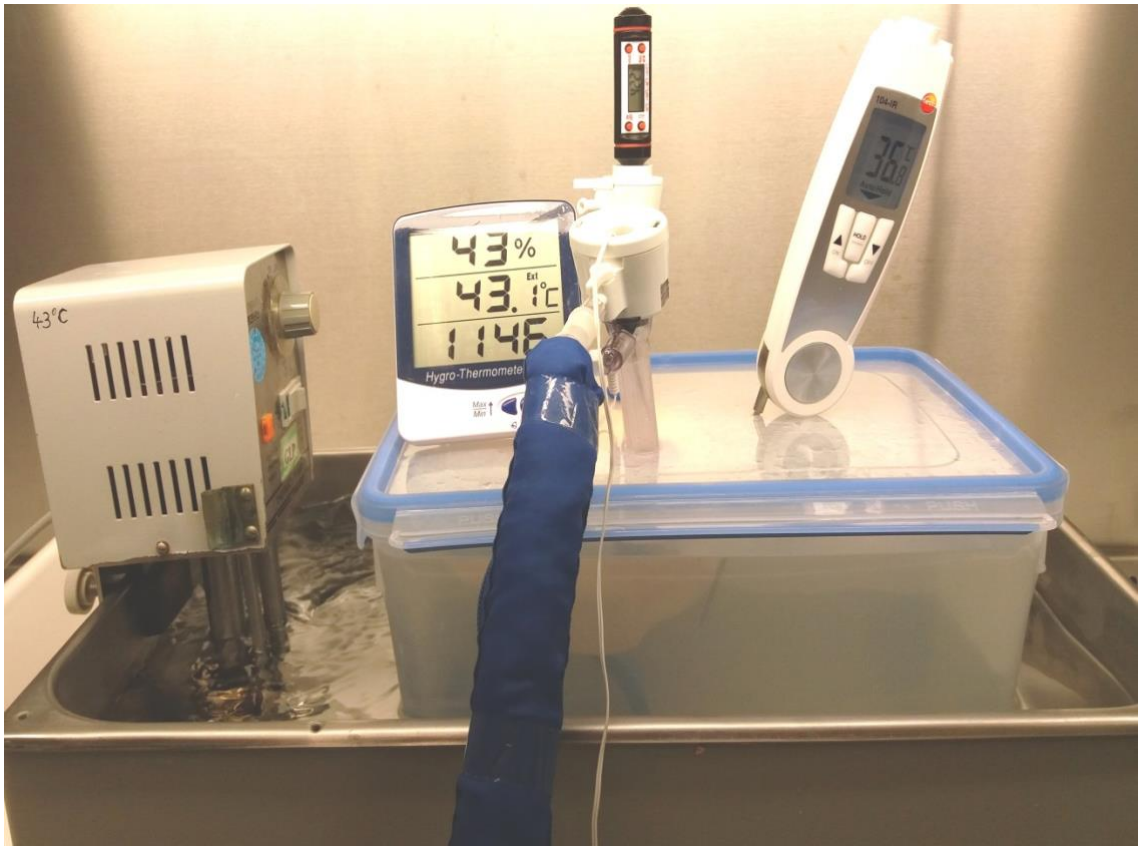

Figure 4: View of the experimental setting using the IBUB ex-vivo model. The IBUB is placed into a closed plastic container, which is immersed into a water bad kept at a constant temperature of 37 °C. Then, the IBUB is heated up with warm-humid CO_2 up (heated inflow tube, blue) until a temperature range between 41 °C – 43 °C is reached. The system is then ready for the heated chemotherapy application.

4.5 Qualitative assessment of the distribution

To establish our hPIPAC model, we first performed a series of experiments in 2 x 4 bladders using solutions stained with 0.0003 % methylene blue to allow visual determination of drug repartition.

4.5.1 Quantitative assessment of drug distribution

For the quantitative experiments we treated ex bladders with the following heated (45°C) drug solutions:

- 2.7 mg doxorubicin (DOXO-cell®, cell pharm GmbH; Bad Vilbel, Germany) in 48,7 ml NaCl and
- 13.5 mg cisplatin (Cisplatin Teva®, TEVA GmbH, Ulm, Germany) in 150 ml NaCl.

These doses and solutions correspond to the quantity applied in a human with a body skin area (BSA) of 2.0 m^2.

4.5.2 Application of therapeutic aerosol

The nebulizer (Capnopen®, Capnomed, Villingendorf, Germany) was inserted into the bladder via a trocar and fixed in position. Then, the nebulizer was connected via a high-pressure line to an angioinjector (Accutron HP®, Medtron AG, Saarbrücken, Germany) equipped with a heating cuff to prevent a fall in temperature during the aerosolization phase (Bachmann et al., 2021). After verifying the tightness of the system, the pre-heated (45 °C) solutions were aerosolized at a flow of 0.6 ml/s at a maximal pressure of 21 bar.

4.5.3 Exposition Time

An exposition time (steady state) of 30 minutes was applied in order to reproduce standard clinical conditions, as described elsewhere (Nowacki et al., 2018).

4.5.4 Exsufflation

After completion of the 30 min exposure, the insufflator was switched off, and the gas was exsufflated safely into a HEPA filter. For further processing, the trocar balloon was first deflated, the suture carefully opened using scissors, and the trocar safely removed.

4.6 Biopsies

The organ was opened and the serosa was examined visually for integrity. Then, standardized 8 mm punch biopsies were taken at the top, the middle and the bottom of the bladder. Biopsies were taken in triplicate at each location and frozen immediately at – 80 °C.

4.6.1 Preanalytical sample preparation for drug concentration measurements

PIPAC tissue samples were prepared for cisplatin (Pt) and doxorubicin measurements as follows:

1. Samples were lyophilized (KF-2-110; H. Saur Laborbedarf, Reutlingen, Germany) in the speedvac and weighed (normalization to "dry weight").
2. The pellets were cut into 4 – 5 little pieces with two scalpels.
3. This step was followed by dissolution in 1 ml Ampuwa water.
4. Samples were homogenized in a miccra-D9 homogenizer (ART-moderne Labortechnik e.V.) for 1 min at room temperature (RT).
5. Samples were sonicated in a sonicater (Elektrosonic type 07) for 20 min at RT.
6. Samples were filled up with 0.5 ml Ampuwa water to give a final volume of 1.5 ml followed by vortexing and centrifugation (cryopreservation at – 80 °C until shipping).

4.6.2 Drug tissue concentration measurements

The tubes with the prepared biopsies were sent to an external, independent, GLP-certified laboratory (Medizinisches Versorgungszentrum, Überörtliche Berufsausübungsgemeinschaft, Dr. Eberhard & Partner Dortmund, Germany) on dry ice. The biopsies had been blinded to the laboratory investigators. For measuring the doxorubicin concentration, a high-performance liquid chromatography (HPLC; Waters Fluorescence Detector 2475, Waters Inc., Milford, MA) was used with a serum LLoQ of 5 ng/ml. Preanalytical validation proved a linear range of measurements in 5 % glucose matrix between 0.1 - 10000 µg/ml doxorubicin and established no influence of organic matrices. An atomic absorption spectroscopy (AAS; ZEEnit P 650, Analytic Jena AG, Jena, Germany) quantified the cisplatin concentration. The lower level of quantification (LLoQ) for platinum was 50 ng/ml (cisplatin 80 ng/ml; calculation factor 1.54). Preanalytical validation proved a linear range of measurements in 5 % glucose matrix between 0.1 - 100 µg/ml platinum and established no influence of organic matrices.

4.6.3 Preanalytical sample preparation for the depth of tissue penetration

The depth of tissue penetration was measured with a fluorescence microscopy (Leica Quantimet Q600). The preparation was as follows: at first, the – 80 °C

frozen biopsies were embedded with a water-soluble embedding medium based on glycerine for cryostat sections at temperatures below – 10 °C, called Tissue Tek (Tissue-Tek, Sacura REF 4583). Afterwards, the biopsies were cut into 10 µm slices in the cryotome (Leica cryocut CM3050S, CT – 20 °C, OT – 21 °C). Subsequently, the sections were pulled on a microscope slide, air-dried, and evaluated by fluorescence microscopy (Leica Quantimet Q 600 with filter: doxorubicin ex 490 nm, abs 560 – 590 nm) (magnitude 10 x). Data were stored and analyzed with Leica software: Leica Qwin 2002.

4.6.4 Immunofluorescence microscopy

Biopsies were first evaluated for tissue integrity using hematoxylin and eosin staining. Samples from 3 x 3 biopsies (top, middle, bottom) were prepared for cryosection (Tissue-Tek, Sacura). Three 10 µm thick sections of each biopsy were cut at a right angle to the surface of the punch biopsy (CT – 20 °C, OT – 21 °C). Sections were fixed with Cytoseal-xyl® on a glass slide and covered. Then, the sections were air-dried at room temperature and analyzed. Measurements were performed using a fluorescence microscope (Leica DMRBE, Wetzlar, Germany) with Leica Qwin 2002 software after initialization and standardization. A picture at magnitude 2.5x was taken in order to get an overview of the sample (size, morphology, completeness of anatomic layers, orientation). Nuclear fluorescence at an emission wavelength of 490 nm and an absorption wavelength between 560 – 590 nm was used to determine the depth of the tissue penetration of Doxorubicin. All microscopic measurements were performed in triplicate by a trained biologist (B.I.) previously trained by a pathologist and blinded to the identity of the samples.

4.7 Occupational health safety

Chemotherapeutics are toxic substances with a high health risk for the practitioner. At the beginning of the year 2016 the laboratories of the National Center of Pleura and Peritoneum were audited successfully by the health insurance of the state of Baden- Wuerttemberg. The access to all research-facilities is limited to the trained employees. All experiments, consisting of the use

of chemotherapeutics, were conducted in a class-2 safety hood certified for application of cytostatic drugs (Maxisafe 2000, ThermoFisher Scientific, Dreieich, Germany). 150 ml NaCl containing 7,5 mg cisplatin (TEVA GmbH, Ulm, Germany) were aerosolized into the safety hood. Potential occupational, environmental contamination was excluded by an independent certified company (DEKRA industrials, Stuttgart, Germany). A Gravikon VC25 device combined with a dust detector (Ströhlein, Kaarst, Germany) were used for the sampling. A cellulose nitrate filter with a diameter of 50 mm with an airflow of 22.5 m^3/h was used the clean the collected air. Toxicological cisplatin levels of research analysis were analyzed according to a standard protocol (NIOSH 7300). The detection limit was 0,3 ng/sample.

Engineers of the Division for Hazardous Substances at the Laboratory for Environmental and Product Analysis of DEKRA industrial GmbH have conducted samples and analysis. Surface contamination is checked at regular intervals. The persons involved are regularly monitored by a specialized physician.

4.8 Sample size

The sample size was calculated on the basis of the data of Khosrawipour et al. (2018) assuming a depth of tissue penetration for Doxorubicin of 348 + / - 47 µm and considering that a difference of less than 20 % between devices would not be clinically meaningful. Further assumptions were an alpha error of 0.05 and a power of 0.8. A sample size of 7 biopsies / per group was determined using an online sample size calculator (Kane, 2020). To take possible sample dropouts into account, a number of nine biopsies in three bladders were chosen.

4.9 Statistics

Descriptive statistics: Continuous data were expressed as mean and confidence intervals 5 – 95 % or, when meaningful, as median values. Comparative statistics were performed using non-parametric tests for comparison of means (PIPAC vs. hPIPAC) and ANOVA with a trend for multiple comparisons (top, middle, and bottom of the bladder). Data were managed and analyzed using SPSS software version 25 (IBM, Chicago, USA).

4.10 Research funding

This research was carried out under a research contract between Fisher & Paykel Healthcare New Zealand (FPH) and the Eberhard Karls Universität Tübingen. The research outline was discussed and agreed upon with FPH. The humidification system prototypes were explicitly developed for this research and made available to the NCPP by FPH. A development engineer from FPH visited the research facility during the experiments and provided counseling on technical issues. FPH did not influence data collection and analysis, the editing of this doctoral thesis, or the peer-reviewed publication in an international journal.

5 Results

5.1 Local tissue toxicity

The first aim of our experiments was to determine if hPIPAC at a temperature between 41 and 43 °C maintained for 30 minutes induces tissue damage.

Firstly, biopsies of the urinary bladder were performed during the different stages of the research: before the experiment, while heating the urinary bladder and after aerosolization of the chemotherapy.

The histological appearance was examined by using hematoxylin-eosin stain and a fluorescence microscope after initialization and standardization. A picture at magnitude 40x was taken in order to get an overview of the sample (size, morphology, completeness of anatomic layers, orientation).

As shown in Figure 5, microscopic analysis showed that the fresh bladders obtained from the slaughterhouse and conserved on ice did not show any sign of tissular or cellular degradation in conventional histology (panel a). There was no evidence of necrosis or edema, the peritoneal mesothelial cells had not shrunk, and the basal membrane was not exposed. There was no peeling of the mucosa and submucosa off the muscle layer.

After heating up the IBUB with warm-humid CO_2, we observed a fluid transfer into the tissue with an accumulation of the aerosolized liquid within the subperitoneal layer, but no cellular edema (panel b). The amount of liquid accumulating between the tissue layers further increased after aerosolization of chemotherapy (cisplatin and doxorubicin diluted into 200 ml NaCl 0.9 % (panel c).

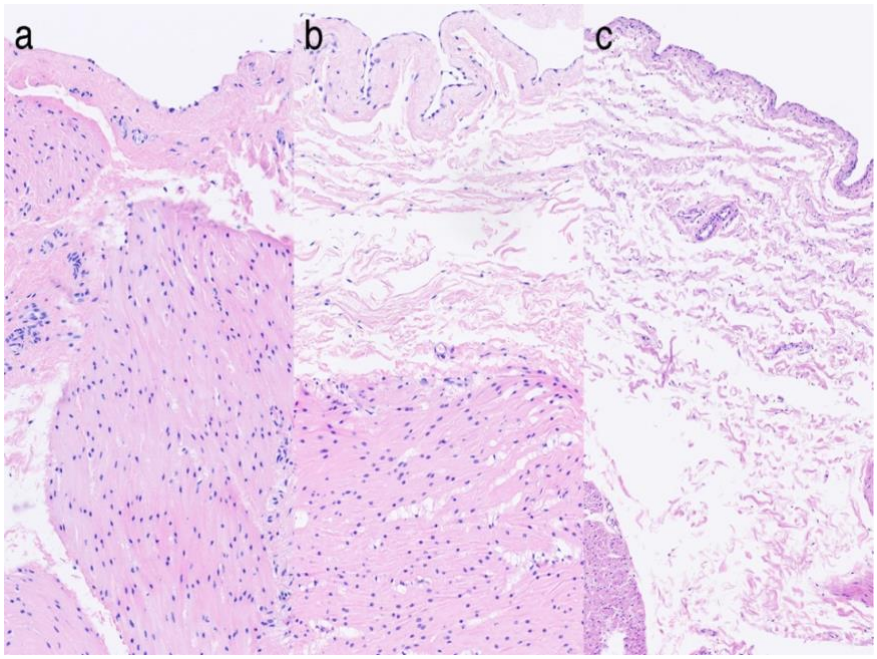

Figure 5: Histological appearance of the inverted bovine urinary bladder (IBUB) before the experiment (control, panel a), after heating up with warm-humid CO_2 (panel b), and after subsequent aerosolization of chemotherapy (cisplatin and doxorubicin diluted in 200 ml NaCl 0.9 %). There is no sign of tissue damage to the untreated organs. During hPIPAC, fluid accumulates in the subperitoneal layer (between the serosa and the muscle) after heating up (panel b) and even more after aerosolization of chemotherapy (panel c). Magnification 40x. Hematoxylin-Eosin (HE) staining.

5.2 Tissue concentration

The second objective was to determine if hPIPAC increases tissue concentration of cisplatin and doxorubicin as compared to PIPAC. The major benefit of intraperitoneal chemotherapy is the regional dose intensity that should be gained. The blinded samples were sent to an external independent laboratory in Dortmund, in view of the fact of the complex examination for detecting the total drug concentration in the tissue. Doxorubicin was measured with a high-performance liquid chromatography and cisplatin was quantified with an atomic absorption spectroscopy. The results of the drugs' concentrations were sent back in tabular form in nanogram per milliliter.

The expectation was to observe an increased drug concentration. Surprisingly, the opposite pattern was observed, namely that tissue drug concentration was reduced after hPIPAC vs. PIPAC. This observation was correct for doxorubicin,

for cisplatin, and at all localizations in the bladder. Table 1 summarizes the average concentration findings from both cytostatic drugs in hPIPAC and PIPAC.

Table 1: Tissue concentration of doxorubicin and cisplatin after hyperthermic PIPAC (test, left column) vs. normothermic PIPAC (control group, right column). Drug concentration is significantly and consistently lower after hPIPAC vs. PIPAC. *Mann-Whitney-U-Test for independent samples.

		hPIPAC (n = 27)	PIPAC (n = 81)	Significance*
Tissue concentration (ng/ml; mean, CI 5 – 95 %)	doxorubicin	8.9 (5.4 – 12.4)	39.5 (31.4 – 47.6)	p < 0.001
	cisplatin	253 (198 – 308)	609 (530 – 687)	p < 0.001

The following figures are meant to clarify statically the different tissue concentrations for hPIPAC (blue) and PIPAC (red) dependent on the biopsy location in the bladder. The bar graph indicates the drug concentration in nanogram per milliliter.

Figure 6 shows the tissue concentration of doxorubicin, depending on the localization in the IBUB. Drug concentration, in all localizations, is higher after PIPAC than after hPIPAC (Kruskal-Wallis H test, p < 0.001).

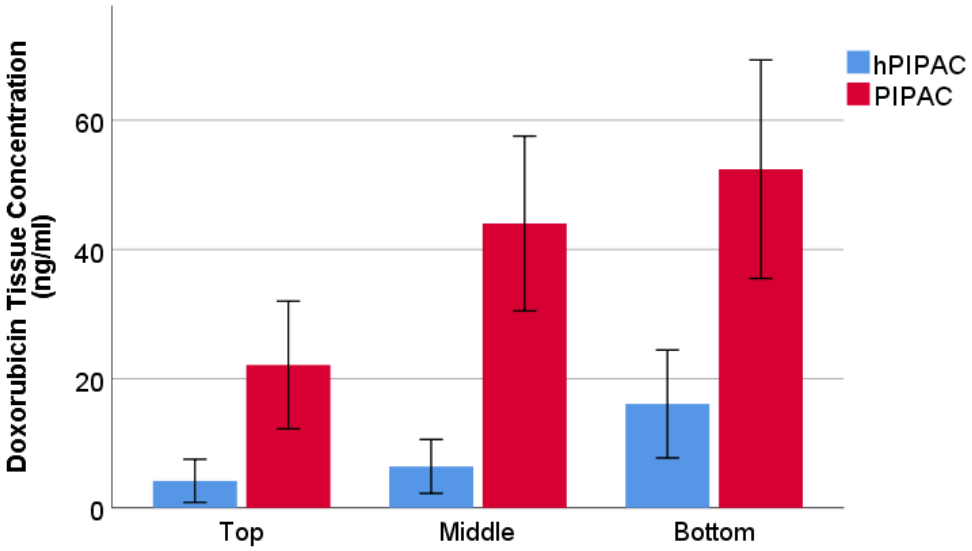

Figure 6: Tissue concentration of doxorubicin after PIPAC vs. hPIPAC. The tissue concentration of doxorubicin depends on the localization in the IBUB, with an increasing gradient from the top to the bottom of the model. However, the tissue drug concentration is consistently higher after PIPAC than after hPIPAC ($p < 0.001$).

Similar data were found when examining the tissue concentration of cisplatin, as demonstrated in Figure 6. The drug concentration of cisplatin was consistently higher after PIPAC than after hPIPAC, in all localizations of the IBUB model (Kruskal-Wallis H test, $p < 0.001$).

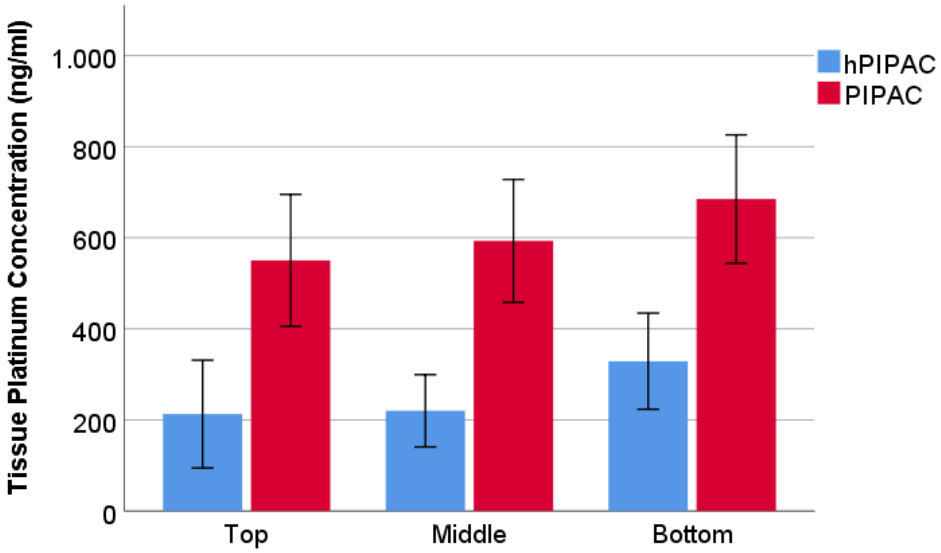

Figure 7: Tissue concentration of cisplatin after PIPAC vs. hPIPAC. The tissue concentration of cisplatin depends on the localization in the IBUB, with an increasing gradient from the top to the bottom of the organ. The tissue drug concentration is consistently higher after PIPAC than after hPIPAC ($p < 0.001$).

The results above are reproducible independently from the bladder examined: there is no significant difference in the tissue concentration of doxorubicin between the different bladders examined, both for PIPAC and hPIPAC (Figure 8).

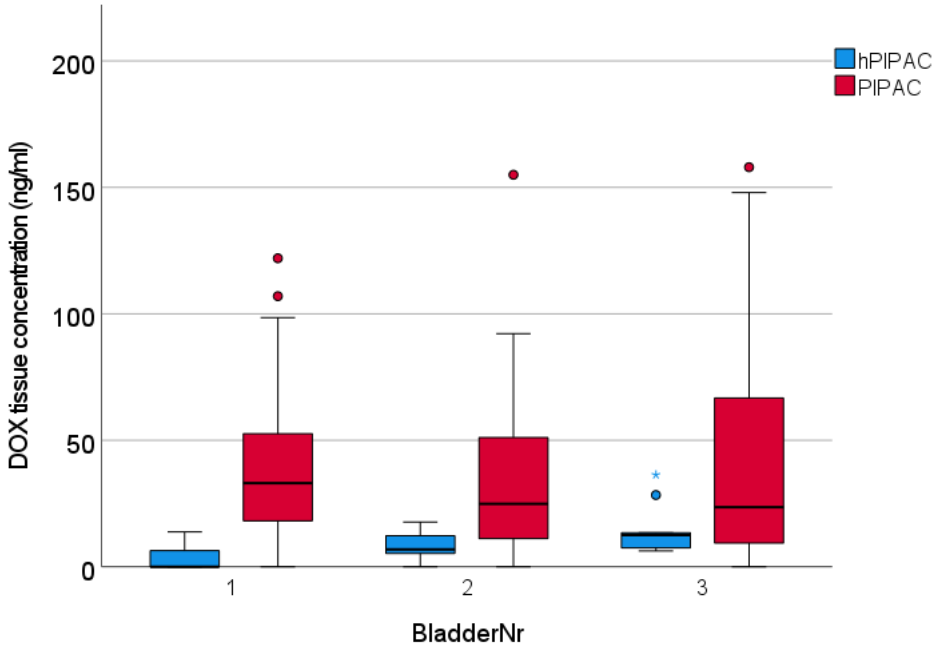

Figure 8: Tissue concentration of doxorubicin in different experiments. The tissue concentration of doxorubicin does not depend on the organ examined (Kruskal-Wallis, p=0.33). The tissue drug concentration is consistently higher after PIPAC than after hPIPAC.

5.3 Depth of tissue penetration

The third objective was to determine if hPIPAC increases the depth of tissue penetration of doxorubicin as compared to PIPAC. To investigate and compare the depth of tissue penetration, white light microscopy is used for the tissue overview (2.5 x Magnification) with Leica Qwin 2002 software after initialization and standardization. For showing the tissue penetration of doxorubicin (here with 20x magnification), nuclear fluorescence at an emission wavelength of 490 nm and an absorption wavelength between 560 – 590 nm was used. Doxorubicin was detected in both groups. Microscopic analysis of both groups showed a

substantial difference in the penetration depth of doxorubicin. The depth was measured in micrometer.

Again, the expectation was to observe an improvement when adding hyperthermia. Qualitatively, the staining after hPIPAC (Figure 9, top panels) was more diffuse than after PIPAC (bottom panels), and nuclear staining was less intense after hPIPAC. Moreover, we observed a liquid infiltration between the tissue layers after hPIPAC.

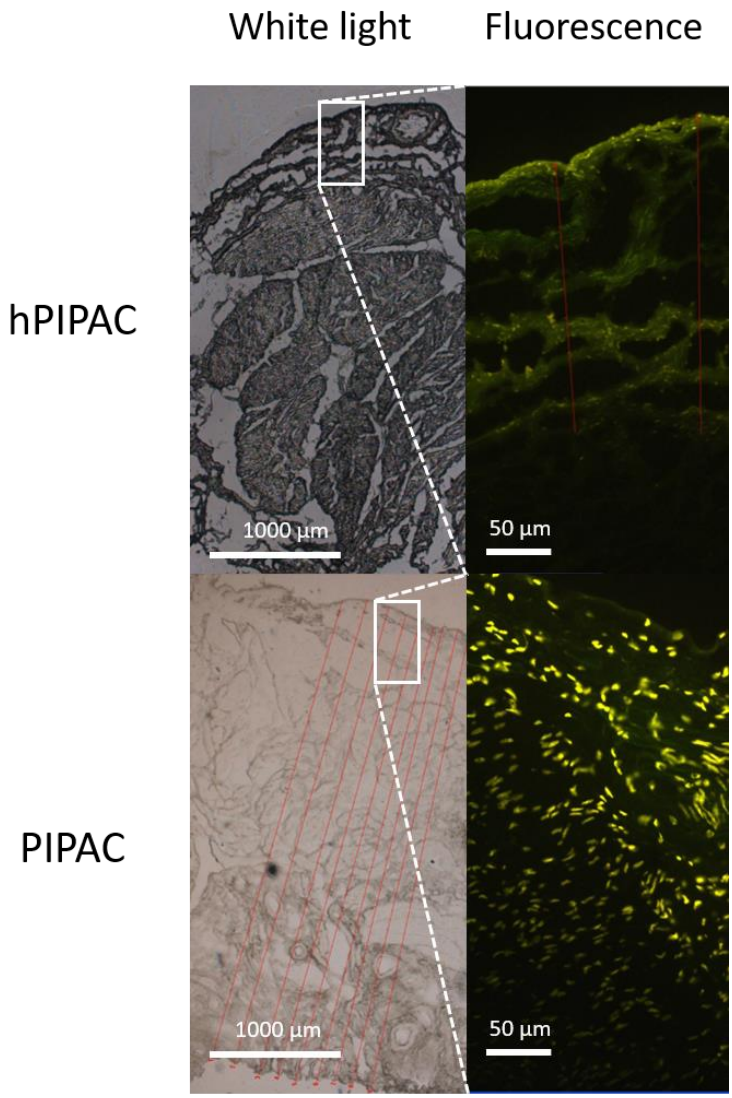

Figure 9: Peritoneal staining pattern after aerosolization of doxorubicin as hPIPAC vs. PIPAC. The left panels show the tissue under white light microscopy; the right panels show tissue staining with doxorubicin by fluorescence microscopy. Magnification: white light 2.5x, fluorescence 20x.

Table 2 summarizes the penetration findings.

Table 2: Depth of tissue penetration of doxorubicin after hyperthermic PIPAC (test, left column) vs. normothermic PIPAC (control group, right column). Drug concentration is consistently lower after hPIPAC vs. PIPAC and approaches statistical significance. *Kruskal-Wallis H test.

Doxorubicin	hPIPAC (n = 198 measurements)	PIPAC (n = 153 measurements)	Significance*
Depth of tissue penetration (µm; mean, CI 5 – 95 %)	504 (273 – 734)	870 (473 – 1326)	P = 0.09

The depth of tissue penetration was consistently lower after hPIPAC, compared to PIPAC. There was some overlapping of the concentration values between groups, which is why the trend observed did not reach statistical significance (p = 0.09, *Kruskal-Wallis H test) in this relatively small sample size.

Figure 10 shows that the difference in the depth of tissue penetration of doxorubicin was more pronounced at the top and the bottom of the model after PIPAC than after hPIPAC.

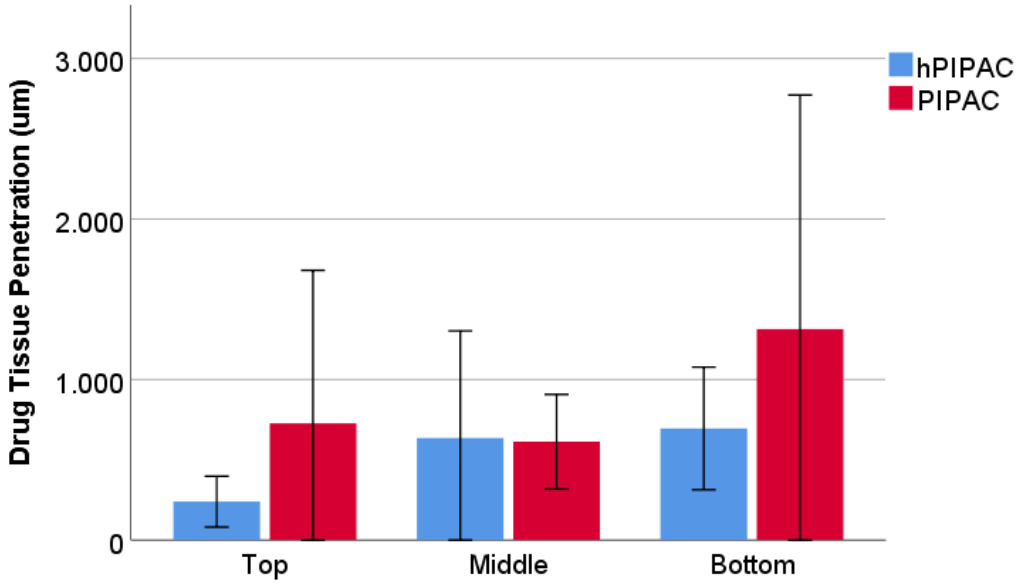

Figure 10: Depth of tissue penetration of doxorubicin after hPIPAC (blue) and PIPAC (red). Tissue penetration is higher after PIPAC at the top and bottom of the model, but not in the middle.

Tissue concentration of doxorubicin and cisplatin are significantly lower after hPIPAC than after PIPAC. The depth of tissue penetration of doxorubicin after hPIPAC is inferior to PIPAC, even if the difference did not reach statistical significance. In any case, there is no superiority of hPIPAC vs. PIPAC regarding the depth of tissue penetration and concentration. Thus, our hypothesis that hPIPAC optimizes the tissue delivery of cisplatin and doxorubicin in the IBUB model can be rejected when the above-mentioned technology is used.

There is some variability in the measurements between the different experiments and, respectively, bladders examined (Figure 11).

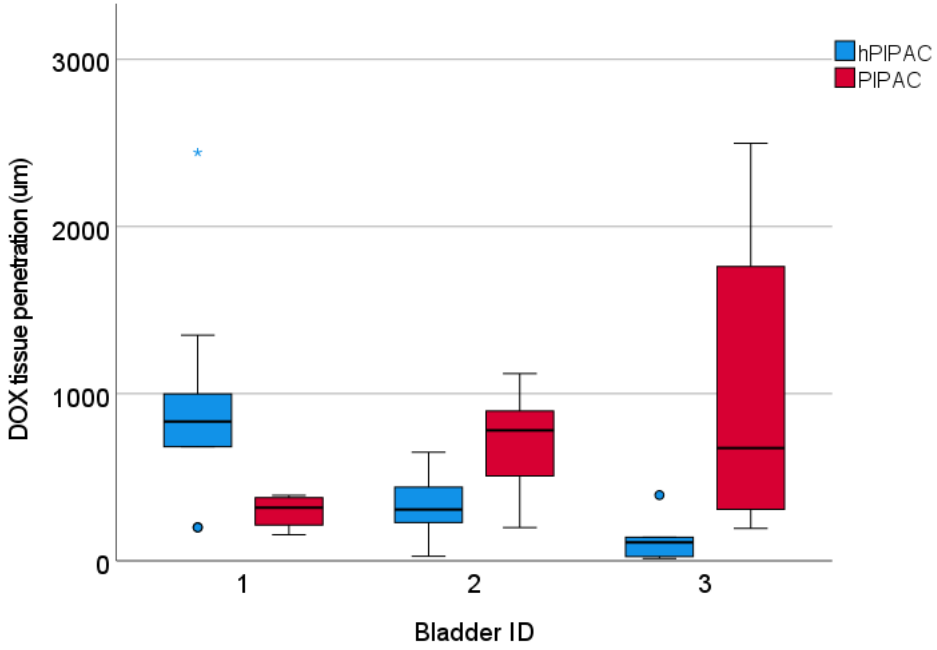

Figure 11: Depth of tissue penetration of doxorubicin after hPIPAC (blue) and PIPAC (red). The measurements of DOX tissue penetration show differences between the individual bladders examined, in particular after PIPAC. However, over all bladders, these differences do not reach statistical significance (p=0.27).

6 Discussion

Current efforts in developing new regional approaches to chemotherapy delivery are a consequence of the relative resistance of peritoneal metastasis to systemic chemotherapy. The rationale of intraperitoneal chemotherapy is that dose intensification through locoregional delivery might provide tumor control by increasing the drug concentrations in the regional tissues (Sugarbaker, 2020).

Previous research suggested that hyperthermia can further optimize the target effect of intraperitoneal delivery of cytostatic drugs. This enhanced effect can be explained by pharmacological or biological factors. Tissue exposure to hyperthermia alters various cellular events, including the rate of apoptosis (Herman et al., 1982). The mechanisms responsible for induced thermotolerance are probably different from those that cause cellular resistance to the cytotoxic effects of the drugs tested (Shen et al., 2012). However, the results were, in part, contradictory. For example, in a healthy swine model, adding hyperthermia during liquid intraperitoneal chemotherapy improved the concentration of platinum in the visceral samples ($p = 0.001$) but not in the parietal peritoneum (Facy et al., 2012).

Our study was first to investigate the pharmacological effect of adding hyperthermia to PIPAC. Our hypothesis was that hPIPAC would increase drug concentration and depth of tissue penetration. This hypothesis has to be rejected, since our results document that hPIPAC does not allow optimizing tissue delivery of cisplatin and doxorubicin in the model tested. The opposite is true: tissue concentration of doxorubicin and cisplatin was significantly reduced by previous heating of the target tissue with warm-humid CO_2. These results might first appear puzzling, but repeated experiments confirmed reproducible results for all organs used for the various localizations within the organs and for both drugs used. There are no previous pharmacological data on hPIPAC. In the only prior publication where PIPAC was combined with hyperthermia (hPIPAC), no drug tissue concentration data were provided (Jung do et al., 2016).

In theory, results might have been different with other chemotherapeutic drugs. The choice of drugs in our study, namely doxorubicin and cisplatin, was dictated by their frequent use for hyperthermic intraperitoneal chemotherapy (HIPEC) (Van der Speeten et al., 2017). The effect of both drugs has been reported to be augmented by the application of hyperthermia (Sugarbaker et al., 2005). Cisplatin is an alkylating agent, and it has been claimed that such agents at elevated temperatures might be the drugs of choice for treating many types of tumors (Takemoto et al., 2003). In Chinese hamster ovary cells in vitro, cis-diamminedichloroplatinum was more toxic at 42.4 – 43 °C than at 37 °C (Herman, 1983). One of the earliest chemotherapy agents used in clinical trials via the intraperitoneal route was doxorubicin. Doxorubicin is an anthracycline that induces double-strand breaks in the tissue and, at higher doses, can provoke significant peritoneal sclerosis due to inflammation (Sugarbaker et al., 2005). Tissue uptake of doxorubicin is enhanced by heat (Jacquet et al., 1996). In contrast, data on a possible thermal enhancement of taxanes (such as paclitaxel and docetaxel), gemcitabine or pemetrexed are conflicting or inconclusive (reviewed in Van der Speeten (2015)). The application of hyperthermia might even be harmful to targeted drugs or biologicals. Antibodies are proteins, and proteins lose their conformation and biological activity at higher temperatures (optimum is 37 °C). In humans, proteins and enzymes can denature when the body temperature is above 40 °C. Thus, it is not surprising that an experimental study evaluating the effects of bevacizumab and hyperthermia in a rodent model of hyperthermic intraperitoneal chemotherapy (HIPEC) delivered negative results (Verhulst, 2013).

Our experiments in the IBUB model do not show any advantage of hyperthermia in enhancing tissue concentration of the chemotherapeutic drugs cisplatin and doxorubicin during PIPAC. They also do not indicate a deeper tissue penetration. In fact, the opposite is true. Our results are in line with those obtained in a rodent model of ovarian cancer where hyperthermic chemoperfusion (42 °C for 60 min) was ineffective in enhancing cisplatin concentration in tumor nodes (Facy et al., 2011).

Thus, based on our results, the potential role of adding hyperthermia to PIPAC by pre-heating the tissue with humid gas should be questioned. A possible explanation for less tissue uptake after hPIPAC is that during the heating phase of the procedure and before chemotherapy application, warm-humid CO_2 was insufflated under pressure into the IBUB, resulting in uptake of liquid tissue in the subperitoneal tissue. This is confirmed by a comparison of histology before heating, between the heating phase and chemotherapy, and after aerosolization of chemotherapy. Tissue morphology shows fluid accumulating in the interstitial space (but no cellular edema). Since tissue uptake of macromolecules through the peritoneal barrier is mainly due to convection and not to diffusion (de Bree et al., 2017), and since interstitial pressure of the target tissue was probably elevated by insufflation of warm-humid CO_2 under pressure during the heating phase, it is reasonable to assume that further liquid uptake (and therefore further drug uptake) was impaired. An indirect confirmation is that the reduction of tissue uptake observed for the administration of doxorubicin with hPIPAC was higher (factor 4.43 in the mean) than cisplatin (factor 2.41). This finding is consistent with the larger molecular weight of doxorubicin (543 g/mol) as compared to cisplatin (300 g/mol).

Our pharmacological experiments with hPIPAC were performed in an ex-vivo model, reproducing many parameters of the clinical situation (Bachmann et al., 2021). Due to its simple geometric shape, our ex-vivo model allows proper evaluation of the homogeneity of the peritoneal coverage, with no differences depending on peritoneal anatomy (ligaments, adhesions, etc.). For example, platin concentration in the pig is varying by a factor up to fifty depending on the anatomical localization (Giger-Pabst et al., 2019), which makes comparisons of different modalities of intraperitoneal chemotherapy in this animal model challenging. Thus, our ex-vivo model has methodological advantages over animal models for optimizing drug delivery techniques to the peritoneum. Moreover, it allows to preserve the life of numerous animals, meeting the 3R-principles (Replacement, Reduction, Refinement (Balls et al., 2009; Russell & Burch, 1959)) expected by regulatory authorities. However, the model choice

might also have influenced our results. The experiments explained above have been performed ex-vivo in post-mortem tissue without blood circulation so that evacuation of interstitial fluid was not possible. In the swine, splanchnic and parietal blood flow is reduced by the hydrostatic pressure applied during laparoscopy (Schafer & Krahenbuhl, 2001). In a rat model, the small and large bowel blood flow was reduced significantly (26.6 % and 23.9 %, respectively). The decreases in the liver, spleen, pancreas, and kidney circulation were 29 – 37 %, 38 – 65 %, 51 – 58 %, and 35 – 41 %, respectively (Schafer et al., 2001). In the human patient, intra-abdominal pressure to 15 mmHg decreased the gastric, small bowel, large bowel, and hepatic blood flow by 40 – 54 %, 32 %, 44 %, and 39 %, respectively. Not only the splanchnic, but also the flow in the parietal peritoneum decreased by 60 % (Schilling et al., 1997). The neovasculature in tumors does not respond to increased temperatures as blood vessels do in normal tissues (Engin, 1994). These differences in blood flow may or may not lead to privileged heating of metastasis in the peritoneal cavity. Thus, drug uptake into the peritoneal tissue after hPIPAC might be considerably different in a living organism and animal experiments will be needed to decide what the further development of hPIPAC may look like.

Another limitation of our study is that only pharmacological and no biological effects of hyperthermia were examined. This aspect is most relevant, since hyperthermia has been proven to delay DNA damage repair and thus to have an intrinsic cytotoxicity. In general, tumor metabolism is acidic (Warburg et al., 1927) and hypoxic (Semenza, 2012). Exposition of colon cancer cells in vitro to CO_2 induced a dramatic intra- and extracellular acidosis (pH 7.4 to 6.2) (Wildbrett et al., 2003). CO_2 insufflation significantly decreased the peritoneal fluid pH in dogs (Duerr et al., 2008), in the rodent (Hanly et al., 2005), and in the swine (Bergstrom et al., 2008). In the context of hPIPAC, acidification of the peritoneal milieu is probably relevant since cancer cells are sensitized to hyperthermia damage by acutely lowering pH. Furthermore, thermotolerance development is reduced at low pH (Engin, 1994).

Acidification of the peritoneal milieu during CO_2-laparoscopy (and therefore during hPIPAC) also induces immunological effects. In vitro, exposition of peritoneal macrophages to CO_2 reduced lipopolysaccharide (LPS)-mediated cytokine release (Hanly et al., 2007). In vivo, acidification of the peritoneal cavity increased serum IL-10 and decreased serum TNF-α in response to the systemic LPS challenge. The degree of inflammatory response reduction was proportional to peritoneal acidification (Hanly et al., 2007). Therefore, all in all, there are numerous biological arguments to further develop hPIPAC.

The technique we used for hPIPAC is only one out of several methods to generate therapeutic hyperthermia (reviewed in Chatterjee et al. (2011)). Local hyperthermia can be generated by external energy sources (e.g. focused ultrasound, infrared and alternating magnetic field), interstitial (e.g. by inserting samples into the tumor under radiological guidance, so-called radiofrequency ablation (RFA)) and nanoparticles (intravenously administered targeted nanoparticles accumulate in tumors and transduce energy delivered externally). Whole body hyperthermia can be reached by placing the patient into a thermal chamber. Regional hyperthermia within the peritoneal cavity is usually generated by extracorporeal circulation or heated liquids (HIPEC, see above).

Innovative formulations can also facilitate the use of hyperthermia for intraperitoneal drug delivery. Novel formulations such as nanomaterials might enable a prolonged residence time of chemotherapeutics in the peritoneal cavity (Dakwar et al., 2017). Moreover, nanomaterials have genuine electrical, optical, magnetic and catalytic properties that might be used to carry and deliver chemotherapeutics specifically into the tumor (Chen et al., 2013). Depending on their structure, nanomaterials can overcome biological barriers such as cell membranes, the gastrointestinal wall, or the blood-brain barrier (Hoet et al., 2004).
For example, the use of elastin-like polypeptides (ELP) has been proposed since ELP accumulate in solid tumors, probably because of the enhanced permeability and retention. Hyperthermia-induced aggregation of ELPs by local heating of the

tumor lead to further enhancement of tumor retention of ELPs (Moktan et al., 2012). Thermal targeting of elastin-like polypeptides increases the potency of doxorubicin, underlying the potential of ELPs in the context of hPIPAC (Zai-Rose et al., 2018).

Nanoparticles can be delivered as PIPAC, hPIPAC or electrostatic precipitation PIPAC (ePIPAC). ePIPAC has been proven to improve spatial homogeneity and enhance tissue penetration of nanoparticles (Van de Sande et al., 2020). Other potential approaches for improving the therapy of peritoneal metastasis are the photodynamic therapy (PDT) and the photothermal therapy (PTT) (Pinto & Pocard, 2018). Photodynamic therapy is a form of phototherapy using light to photosensitize chemical molecules. Photosensitizers are first given intravenously and accumulate in the peritoneal tumor nodes, as a result of their tumor-specific morphology and receptor-specific characteristics. When high-energy photons (usually a laser beam) are distributed within the peritoneal cavity, they are absorbed by the tumors, leading to stimulated energy states of the sensitizer. Reactive oxygen species are generated, and these free radicals are capable of destroying vital cellular structures such as membranes. In addition to cellular damage, tumor vascularization collapses just minutes after being exposed to light. Alternatively, electromagnetic radiation (most often in infrared wavelengths) can be used for the treatment of cancer, the so-called photothermal therapy (PTT). Both PDT and PTT are promising combination therapies for preventing or treating peritoneal metastasis.

In preclinical PDT models, new-generation photosensitizers showed a better tumoral biodistribution and induced a significant survival advantage when added to cytoreductive surgery. These results could lead to promising developments. Nonetheless, it is necessary to test these new photosensitizers in clinical studies to confirm the preclinical results and to verify the tolerance and effectiveness of this therapy.

Photothermal therapy (PTT) is a newer therapeutic treatment in oncology. At the present time, only a few articles analyze the effectiveness of PTT compared to PDT in peritoneal metastasis treatment (Pinto & Pocard, 2018). First preclinical

PTT studies with injected gold nanoparticles and the use of near infrared light on mice demonstrated a regression of tumoral growth and higher survival rates compared to the control groups (Pinto & Pocard, 2018; Wu et al., 2015).

Since the technology we developed for hPIPAC is using an infrared energy source, the logical next step in research would be to combine our hPIPAC technology with local administration of aerosolized nanoparticles for treatment of peritoneal metastasis with PTT.

7 Conclusion

Peritoneal metastasis is relatively resistant to systemic chemotherapy and has a poor prognosis. The therapy remains palliative with the aim of prolonging life and preserving its quality. There is a need to develop improved new therapeutic approaches. An example for a new drug delivery system that addresses systemically the known limitations of intraperitoneal chemotherapy for treating peritoneal metastasis has been pressurized intraperitoneal aerosol chemotherapy (PIPAC). Preclinical experiments, patient cohorts, and controlled clinical studies suggest that PIPAC might be a significant step forward to improve the efficacy of intraperitoneal chemotherapy.

Prior scientific research showed that the addition of hyperthermia to chemotherapy can increase its cytotoxic efficacy. Our research hypothesized that the addition of hyperthermia would further improve the pharmacological properties of PIPAC. In this study, we used a validated prototype for establishing and maintaining tissue temperature within a range of 41 – 43 °C in an ex-vivo organ model. The specific aim was to determine whether hyperthermia can increase drug tissue concentration as well as the depth of tissue penetration, as compared to normothermic PIPAC.

The drugs cisplatin and doxorubicin were aerosolized into a test group (hyperthermia, n = 3 organs) and a control group (normothermia, n = 9 organs) of fresh inverted bovine urinary bladders (IBUB) obtained from the slaughterhouse. The CO_2 filled IBUB has an equivalent volume to the expanded human abdominal cavity, and then its inner surface is overlaid with peritoneum. Altogether, 108 biopsies were taken at standardized locations and prepared for further analysis.

Pharmacological measures were performed in a GLP-certified laboratory. There, doxorubicin concentration was measured by high-performance liquid chromatography and cisplatin concentration was quantified by atomic absorption spectroscopy. The depth of tissue penetration of doxorubicin was determined using a fluorescence microscope in our laboratory. All analyses were blinded.

Surprisingly, results showed no significant pharmacological advantage of hPIPAC over PIPAC. Neither the tissue concentration of doxorubicin nor cisplatin was enhanced by therapeutic hyperthermia. Doxorubicin did not penetrate the tissue more deeply under hyperthermic conditions.

A possible explanation for this negative result is a significant liquid uptake by the target tissue during the warming phase preceding the application of chemotherapy. We confirmed this liquid uptake by histology. As a result, we hypothesize that interstitial fluid pressure increases within the peritoneum and the retroperitoneal tissue, which is an obstacle to drug tissue uptake.

The interpretation of these results should be done cautiously. First, we only evaluated the pharmacological effects and not the biological impact of hyperthermia on the target tissue. Second, we used ex-vivo, post-mortem tissue. Finally, although the model used simulated heat loss, there was no blood circulation.

Further studies are needed before it is possible to conclude that hPIPAC has no pharmacological or biological advantage over PIPAC. These experiments should include measurements in a living animal model. Possibly, other drug delivery technologies might overcome current limitations and allow physicians to exploit the full potential of hyperthermia in combination with PIPAC.

8 Zusammenfassung in deutscher Sprache

Peritonealmetastasen haben eine schlechte Prognose. Die Therapie ist in der Regel palliativ mit dem Ziel, das Leben zu verlängern und die Lebensqualität zu erhalten. Peritonealmetastasen sind relativ resistent gegenüber systemischer Chemotherapie. Folglich besteht ein großes Interesse an der Entwicklung innovativer Chemotherapieformen, wie zum Beispiel durch die intraperitoneale Abgabe der zytotoxischen Substanzen. Ein solcher neuer Ansatz ist die intraperitoneale Druck – Aerosol – Chemotherapie (*Pressurized Intraperitoneal Aerosol Chemotherapy (PIPAC)*). Die PIPAC erlaubt eine teilweise Überwindung der bekannten pharmakologischen Einschränkungen der intravenösen Chemotherapie bei Behandlung von Peritonealmetastasen. (Prä-) Klinische Studien und Patientenkohortendaten legen nahe, dass die PIPAC ein sicheres Verfahren ist und die Regression von chemoresistenten Peritonealmetastasen induzieren kann, ohne die Lebensqualität der Patienten wesentlich einzuschränken.

Frühere wissenschaftliche Untersuchungen haben gezeigt, dass die Zugabe von Hyperthermie zur Chemotherapie die zytotoxische Wirksamkeit erhöhen kann. In dieser Arbeit wurde die Hypothese geprüft, ob die pharmakologischen Eigenschaften von PIPAC unter hyperthermen Bedingungen (sog. hPIPAC) weiter verbessert werden. In diesem Zusammenhang wird in dieser Studie ein Medizingerätprototyp zur Generierung und Aufrechterhaltung einer Hyperthermie im ex-vivo Zielgewebe validiert. Spezifisch wird geprüft, ob die Konzentration der Arzneimittel, sowie die Tiefe der Durchdringung im Peritonealgewebe im Vergleich zur normothermem PIPAC erhöht ist.

Für sämtliche Untersuchungen dienten frische, invertierte Rinderharnblasen. Rinderblasen haben ein ähnliches Volumen wie die menschliche Bauchhöhle und nach Einstülpen des Organs ist ihre innere Oberfläche mit Peritoneum ausgekleidet. Die invertierten Harnblasen sind mit den aerosolisierten Arzneimitteln Cisplatin und Doxorubicin unter einem Druck von 12 – 15 mmHg

und Hyperthermie – Bedingungen (41 – 43 °C) für 30 Minuten behandelt worden. Eine Testgruppe (Hyperthermie, n = 3) und eine Kontrollgruppe (Normothermie, n = 9) wurden verglichen. Insgesamt wurden 108 Biopsien an standardisierten, räumlich repräsentativen Lokalisationen entnommen und für die weiteren Analysen vorbereitet.
Die pharmakologischen Gewebekonzentrationsmessungen erfolgten durch ein externes, GLP-zertifiziertes Labor. Dabei wurde die Doxorubicin-Konzentration durch Hochleistungsflüssigchromatographie (HPLC) bestimmt und die Cisplatin-Konzentration durch Atomabsorptionsspektroskopie quantifiziert. Die Tiefe der Gewebedurchdringung wurde am Beispiel von Doxorubicin mittels direkter Fluoreszenzmikroskopie in unserem Labor bestimmt. Alle Analysen waren verblindet.

Unsere Ergebnisse zeigen keinen signifikanten pharmakologischen Vorteil von hPIPAC gegenüber PIPAC. Weder die Gewebekonzentration von Doxorubicin noch die von Cisplatin wurde durch therapeutische Hyperthermie erhöht. Doxorubicin drang unter hyperthermischen Bedingungen nicht tiefer in das Gewebe ein.

Eine wahrscheinliche Erklärung für dieses überraschende negative Ergebnis ist eine signifikante Flüssigkeitsaufnahme durch das Zielgewebe, die schon während der Erwärmungsphase und vor der Anwendung der Chemotherapie histologisch belegt werden konnte. Wir stellen daher die Hypothese auf, dass der interstitielle Flüssigkeitsdruck im Peritoneum und im retroperitonealen Gewebe schon vor der Applikation der Chemotherapie ansteigt. Da Makromoleküle vor allem durch Konvektion in das Gewebe transportiert werden, bedeutet ein erhöhter interstitieller Flüssigkeitsdruck ein physikalisches Hindernis für die Aufnahme von Arzneimitteln.

Diese Ergebnisse sollten mit Vorsicht interpretiert werden. Erstens untersuchten wir nur die pharmakologischen Wirkungen und nicht die biologischen Auswirkungen der Hyperthermie im Zielgewebe, zweitens verwendeten wir Post-

Mortem-Gewebe. Obwohl ein Wärmeverlust simuliert wurde, gab es keine Gewebedurchblutung.

Weitere Studien sind erforderlich, bevor geschlussfolgert werden kann, dass die hPIPAC keinen pharmakologischen oder biologischen Vorteil gegenüber der PIPAC hat. Diese Experimente sollten mit Messungen in einem lebenden Tiermodell einhergehen. Möglicherweise könnte eine optimierte Technologie die derzeitigen Einschränkungen in der Arzneimittelabgabe überwinden und es ermöglichen, das volle Potenzial der Hyperthermie in Kombination mit der PIPAC auszuschöpfen.

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10 Erklärung zum Eigenanteil

Diese Arbeit wurde in der chirurgischen Universitätsklinik Tübingen, Klinik für Allgemeine, Viszeral- und Transplantationschirurgie unter der Betreuung von Herrn Professor Dr. Alfred Königsrainer, sowie unter der wissenschaftlichen Anleitung durch Herrn Professor Dr. Marc André Reymond durchgeführt.
Die Konzeption der hyperthermen PIPAC lag in meiner Verantwortung. Bei der Entwicklung dieser Studie unterstützte mich Professor Reymond.

Die Versuchsaufbauten und Testexperimente erfolgten durch meine Person eigenständig. Der finale Versuchsaufbau, der zu den Ergebnissen dieser Arbeit geführt hat, entstand in Kooperation mit Frau Christine Bachmann.

Nach Einweisung in das Labor, sowie in das Equipment, durch Herrn Dr. hum. biol. Jürgen Weinreich, Herrn Professor Reymond und Herrn Iaroslaw Sautkin, führte ich die Experimente selbstständig durch.

Bei der Aufarbeitung der Proben für die Gewebekonzentrationsmessung der Arzneimittel unterstütze mich Herr Dr. Weinreich. Die Konzentrationsmessungen von Cisplatin und Doxorubicin erfolgten durch das externe Labor Dr. Eberhard & Partner in Dortmund. Die Ergebnisse wurden mir zur weiteren Datenauswertung in tabellarischer Form zugesandt.

Die gesamte Datenauswertung lag hierbei in meiner Verantwortung, unterstützt wurde ich dabei durch Professor Reymond.

Hiermit versichere ich, Felicitas Giuliana Held, dass ich die vorgelegte Dissertationsarbeit selbständig und ohne unzulässige fremde Hilfe verfasst habe, keine anderen als die ausdrücklich bezeichneten Quellen und Hilfsmittel verwendet habe und wörtlich oder inhaltlich übernommene Stellen von mir als solche gekennzeichnet worden sind.

Weiterhin erkläre ich, dass die digitalen Abbildungen, die Diagramme und die Tabellen, nur die originalen Daten enthalten und in keinem Fall Inhaltsveränderungen vorgenommen wurden.

Tübingen, den 30.09.2022

Felicitas Giuliana Held

11 Danksagungen

Mein Dank gilt zuallererst Professor Dr. Alfred Königsrainer, dem ärztlichen Direktor der Klinik für Allgemeine, Viszeral- und Transplantationschirurgie Tübingen, für die Möglichkeit, in diesem großartigen Team zu promovieren.
Danach möchte ich mich herzlich bei Professor Dr. Marc André Reymond für die Vergabe dieses interessanten Projektes, die Betreuung, die fortwährende Unterstützung, die Diskussionsbereitschaft und für die Ermöglichung der Teilnahme an internationalen Konferenzen bedanken. Die zahlreichen Gespräche auf intellektueller sowie persönlicher Ebene werden mir immer als bereichernder, konstruktiver Austausch und mit einem Lächeln in Erinnerung bleiben.
Dem kompletten Team des „National Center for Pleura and Peritoneum“ gilt mein Dank, hierbei insbesondere Dr. Jürgen Weinreich, Dr. Christine Bachmann, Dr. Wiebke Solass und Dr. Iaroslaw Sautkin.

Ebenso danke ich all denen (insbesondere meiner wunderbaren Familie), die beruflich und privat stetig an meiner Seite standen.

Gewidmet, in tiefer Dankbarkeit, ist diese Arbeit zwei besonderen Frauen: meiner Mama Dr. Monika Held und meiner verstorbenen Omi Hildegard Krüger.
Mama, ohne Deine Unterstützung wäre mein Studium, und somit auch diese Arbeit, nicht möglich gewesen. Danke Dir für Deinen unermesslichen Rückhalt.
Omi, Du hast mir Zeit Deines Lebens Zuversicht, Mut, Lebensfreude, Vertrauen und unendlich viel Liebe geschenkt.