Research internship

Willem van Doesum

Holmium therapy
Controlled release from liposomes by HIFU

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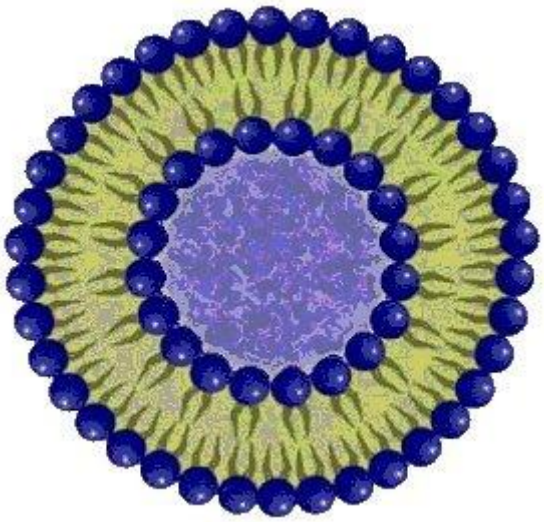
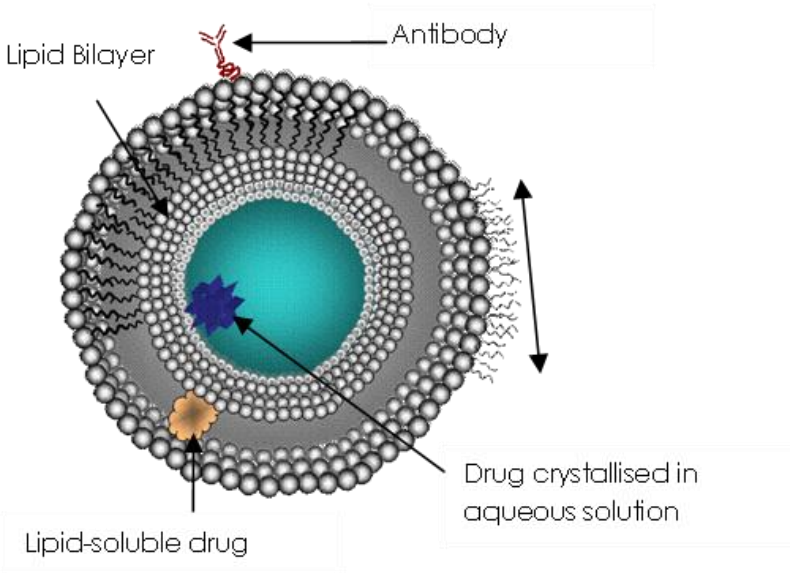
Controlled release from liposomes by HIFU





Summary

Triggered release from liposomes has mainly been reported for hydrophilic compounds. Unfortunately, the largest group of anti-cancer therapies involve agents that are lipophilic. This study proposes a high-intensity focused ultrasound (HIFU)-triggered release system for both lipophilic and hydrophilic compounds from liposomes.

Thus, a thermosensitive liposome formulation was prepared, characterized and subsequently exposed to HIFU at different acoustic powers and time durations. The liposome size was around 130 nm (PDI <0.02) and the T_m of both liposome formulations was around 42 ^oC.

Furthermore, a non-thermosensitive liposome was developed. The liposome size was around 100 nm (PDI <0.07) and no T_m was measured.

After exposure of both liposome formulations to HIFU, an increase release was observed when the acoustic power or exposure duration was increased. Fluorescein had its highest release after 16 minutes at 80 W (90%). Nile Red showed 60% release after 16 minutes of exposure at 80 W.

After HIFU exposure, the liposome characteristics were not affected.

This study demonstrates ultrasound-triggered release of a substantial amount of lipophilic compounds from liposomes. The exact mechanism of this release requires further study. The flexibility of the acoustic power and exposure durations promises a controllable release. It shows great promise for future drug delivery systems.

Introduction

The conventional cancer therapies involve systemic administration of cytotoxic compounds. Currently, the focus is shifting towards a more local approach use of these therapeutics using for instance Drug Delivery Systems.[1] A more local treatment of the affected site would ideally ensure less exposure of healthy tissue to cytotoxic compounds, resulting in a reduction of systemic side effects.

Nano-sized particles have the ability to facilitate passive drug delivery by the enhanced permeability and retention effect in tumours.[2] This would mean that, through accumulation, a higher dose of therapeutic substance can be achieved on the site where the effects are needed.

Liposomes are nanocarriers which hold promises for drug delivery systems. Liposomes are spherical particles with a phospholipid bi-layer surrounding an aqueous core in which hydrophilic drugs can be dissolved. In the phospholipid bi-layer of the liposomes, lipophilic drugs can be solubilised.[3]

By using different components in the phospholipid bi-layer, different types of liposomes can be created. By introducing phospholipids displaying a phase transition temperature (T_m) of 41-42 ^oC in the liposomal bi-layer, thermosensitive liposomes (TSL) can be created. When the phase transition temperature is reached, the phospholipids change from a solid gel to a liquid disordered phase. Consequently, the permeability of the liposomal membrane is significantly enhanced and the encapsulated drug is released.[4]

However, this concept is only applicable to hydrophilic compounds which are dissolved in the aqueous liposomal core. Lipophilic compounds are bound to the hydrocarbon chains of the phospholipids by the van der Waals force.[5] The van der Waals forces are not compromised by the conformational change after reaching the phase transition temperature. Thus lipophilic compounds will not be released from liposomes using only temperature elevation.

However, many useful drugs have a lipophilic nature. Therefore, a technology needed to be developed that would be able to release lipophilic drugs from liposomes.

High Intensity Focused Ultrasound (HIFU) is a technique that is able to facilitate triggered drug release from different drug delivery systems.[6] HIFU has two mechanisms of action. Firstly, HIFU results in the induction of hyperthermia due to the absorption of the energy. Secondly, HIFU causes cavity-formation in liquids that implode subsequently resulting in shear forces.[6, 7]

It is hypothesized that the mechanical forces resulting from cavitation may facilitate the disruption of the liposomal bi-layer and subsequently facilitate release of both hydrophilic and lipophilic compounds from liposomes.

The aim of this study was to investigate the release of both hydrophilic and lipophilic compounds from liposomes by HIFU. To do this, hydrophilic or lipophilic dyes were included in both thermosensitive and a non-thermosensitive liposomes. These liposomes were exposed to HIFU at different wattages for different times and the release was measured.

Matherials and methods

Materials

All chemicals and lipids were commercially available and used as obtained. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG_{2000}-DSPE) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (>99%) and Nile Red (Standard Fluka) were supplied by Sigma Aldrich (Steinheim, Germany). 1,2-distearoyl-*sn*-3-glyceryl-phosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Sodium fluorescein (100 mg/mL) was obtained from Serb Laboratories (Paris, France). Slide-A-Lyzer cassettes (MW cut-off 3,5 kDa) were obtained from Pierce (Rockford, IL, USA). Microbubbles (SonoVue) were obtained from Bracco (Milano, Italy).

Liposome preparation

Fluorescein-containing (F-TSL), Nile Red-containing thermosensitive liposomes (NR-TSL), fluorescein-containing non-thermosensitive liposomes (F-NTSL) and Nile Red-containing non-thermosensitive liposomes (NR-NTSL) were prepared with the conventional thin-film hydration. The TSL formulations consisted of DPPC, DSPC, cholesterol and PEG_{2000}-DSPE in a molar ratio of 67 : 15 : 13 : 5. The NTSL formulations consisted of DSPC, cholesterol and PEG_{2000}-DSPE in a molar ratio of 56 : 39 : 5.
Nile Red was introduced into the phospholipid bi-layer in a concentration of 125 mmol/mol. The lipid mixtures were dissolved in ethanol (10 mL) and evaporated to dryness by rotary evaporation under vacuum (Rotavapor R-210, BUCHI Laboratory Equipment, Zurich, Switzerland). The resulting lipid films were further dried under N_2 to ensure that the ethanol essentially had evaporated. For NR-TSL and NR-NTSL, the lipid film was hydrated in 10 mL HEPES-buffered saline (HBS, 20 mM HEPES and 135 mM NaCl, pH 7.4). For F-TSL and F-NTSL, 10 mL fluorescein (25 mg/mL) was used to hydrate the lipid film. The resulting lipid dispersions were sized with sequential extrusion using a Lipex Extruder (Northern Lipids Inc., Vancouver, Canada) and polycarbonate membrane filters (Poretics Corporation, Livermore, CA) with a pore diameter of 600, 400, 200 and 100 nm to obtain liposomes with an average diameter of around 130 nm. Non-encapsulated fluorescein (F-TSL and F-NTSL) was removed by dialysis in HBS at room temperature using Slide-A-Lyzer cassettes during 48 h with four times a change of buffer.

Liposome characterization

The average hydrodynamic size and polydispersity index (PDI) of freshly prepared liposomes and liposomes after heat- or HIFU exposure were determined with dynamic light scattering (DLS) using a Malvern ALV CGS-3 system (Malvern Instruments Ltd., Worcestershire, United Kingdom). The PDI value can range from 0 for a monodisperse to 1 for a heterodisperse formulation. Intensity correlation functions were measured using a wavelength of 632.8 nm at a scattering angle of 90°. Differential scanning calorimetry (DSC) measurements were performed in a capillary cell microcalorimeter instrument (MicroCal VP-DSC, Northampton, MA) to determine the phase transition melting temperature (T_m) of the liposomes. The experiment was performed at temperatures ranging from 25 to 60°C at a heating rate of 1°C min^{-1} after an equilibration period of 5 min at 25°C. All experiments were performed with liposome dispersions with and without exposure to HIFU.

Ultrasound-triggered release studies

For (pulsed) HIFU-triggered release of Nile Red and fluorescein, an in-house developed HIFU system was used (Fig. 1). At the position of the focal point, a sample volume of 0.5 mL liposome dispersion was placed in a sample holder prepared from a Slide-A-Lyzer cassette, which was adapted before usage to prevent interference of the HIFU beam with the sample holder and avoid disturbance of the focal point. Therefore, the outer plastic surrounding of the cassette including the membrane was removed. The remaining silicone-like gasket was sealed with a clear polyester heat seal membrane (Waters, Acquity UPLC Consumables) and filled with 0.5 mL of liposome dispersion.
As much excess air as possible was removed from the sample holder using a syringe. The samples were subjected to a continuous wave with a frequency of 1.5 MHz at different acoustic powers (0, 10, 20, 40, 80, 120 W) for 4 minutes and to different time periods (1, 2, 4, 8 and 16 minutes) at 80 W of acoustic power. For pulsed HIFU, a time series of 0.5, 1, 2, 4, 8, 16 and 32 at 80 W of acoustic power was used at a duty cycle (DC) of 10% (100 µsec) within a PRP of 1 msec. When microbubbles (7%) were added to the liposome dispersions, exposure times were reduced to a maximum of 8 min, since it was expected that the microbubbles would collapse immediately after the onset of HIFU exposure. The core temperature of the water bath containing the HIFU transducer was set to 37°C for all experiments. For F-TSL, additionally the HIFU measurements were repeated at a core temperature of 20°C, to distinguish temperature-triggered fluorescein from HIFU-triggered fluorescein release.
The temperature at the position of the focal point was measured with a thermocouple fixed in the sample compartment. Fluorescence intensity of the exposed samples was measured using a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm for F-TSL and F-NTSL. For NR-TSL and NR-NTSL, an excitation wavelength of 550 nm and an emission wavelength of 600 nm was used. All experiments were performed in triple.

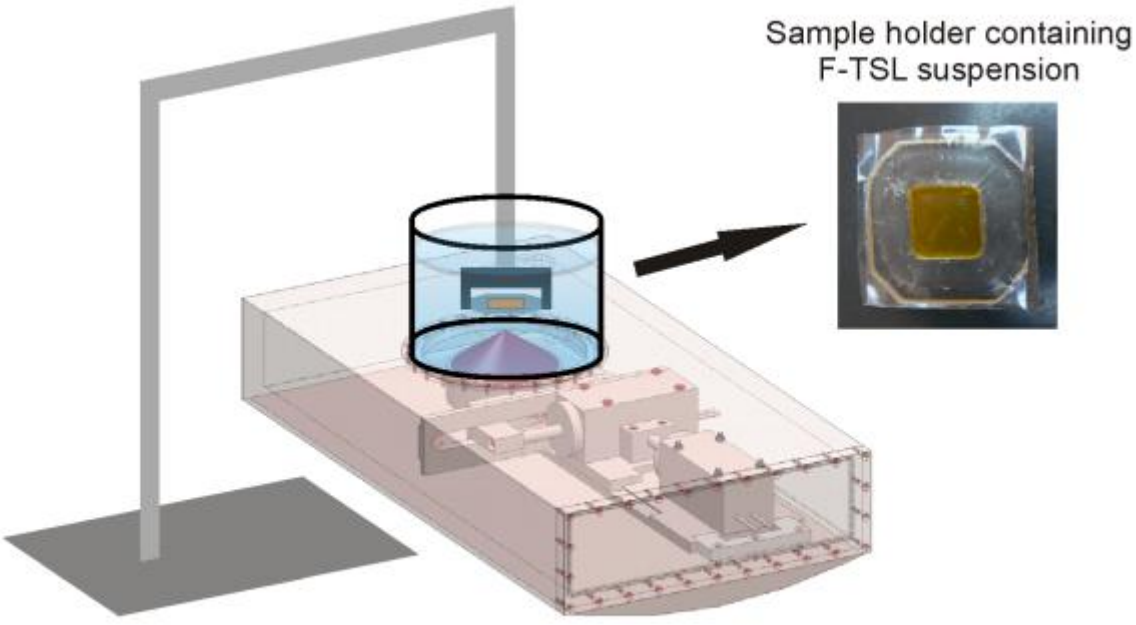


Fig. 1: HIFU system used for ultrasound exposure to the liposome formulations. The sample holder (filled with liposome suspension) was placed in the focal point above the ultrasound transducer.

Measurements

For calculating the release of fluorescent dyes from the liposomes the following information was used.
Nile red is most fluorescent when inside the liposome. When solubilised in water, the fluorescense is quenched. Hence, unprocessed liposomes filled with Nile Red were used as a 100% marker. The loss of fluorescense was used as a measurement for the release of Nile Red from the liposomes.
Fluorescein is most fluorescent outside of the liposome. Hence, unprocessed liposomes filled with Fluorescein were used as a 0% marker. In order to properly calculate release, a 100% marker was necessary. By treating these liposomes with Triton X-100 and heating them, the 100% marker was created. The comparison between these parameters was used to calculate the release of Fluorescein from the liposomes.

Results & Discussion

Liposome characteristics

Size, PDI and T_m of the liposomes were measured directly after preparation (Table 1). All 4 liposome formulations were considered monodisperse with a size around 100 – 130 nm and a PDI ≤ 0.07. The T_m of F-TSL and NR-TSL was 42.1 and 42.3°C, respectively. For F-NTSL and NR-NTSL, no T_m was detected. Furthermore, mean size and PDI after heating the liposomes in HBS or HBS:FCS (1:1, measured at 20°C) did not significantly change as compared to non-heated liposomes, which was also observed after all HIFU exposure experiments of the different liposome formulations (data not shown).

Liposome formulation	Size (nm)	PDI	T_m (°C)
F-TSL	133	0.02	42.1
F-NTSL	97	0.05	n/a*)
NR-TSL	139	0.01	42.3
NR-NTSL	103	0.06	n/a*)

Table 1: Size, PDI and T_m of all 4 liposome formulations. *) No T_m was not found within the measured temperature range (25 to 60°C).

Continuous wave HIFU-triggered release

F-TSL and F-NTSL

Fig. 2 shows the release of fluorescein from F-TSL and F-NTSL after HIFU exposure at different acoustic powers up to 120 W for 4 min (a) and at 80 W for different time durations up to 16 min (b). F-TSL instantly showed an almost complete release, which can be explained by the temperature elevation. The temperature measured within the focal spot of the sample holders indicated a mean temperature of 44 °C at 4 min exposure to 10 W and increasing temperatures with increased wattages. All temperatures exceed the liposomal T_m, causing the direct release of fluorescein. A comparable release was seen after HIFU exposure to 80 W at different time durations. To investigate whether a factor besides temperature influenced fluorescein release from F-TSL existed, the measurements were repeated with the water bath containing to HIFU transducer adjusted to 20°C. It was observed that the liposomal T_m was only exceeded when the acoustic power was set to 80 W (44.5 °C after 4 min of exposure time). Since release was observed before the temperature threshold was exceeded, a different release mechanism below the temperature threshold was strongly suggested.
Furthermore, F-NTSL, which do not have a liposomal T_m and therefore remain stable after temperature elevation, also show substantial fluorescein release after HIFU-exposure. This confirms the presence of an alternate release mechanism apart from temperature-triggered release.
The release from F-NTSL, however, was considerably lower as compared to F-TSL. This can be explained by the difference in cholesterol fraction within the liposomal bi-layer, which was higher in F-NTSL (39% versus 13 %). Cholesterol can stabilize and rigidify the phospholipid bilayer[8], which makes the NTSL less susceptible to destabilization resulting in a decreased release as compared to TSL.

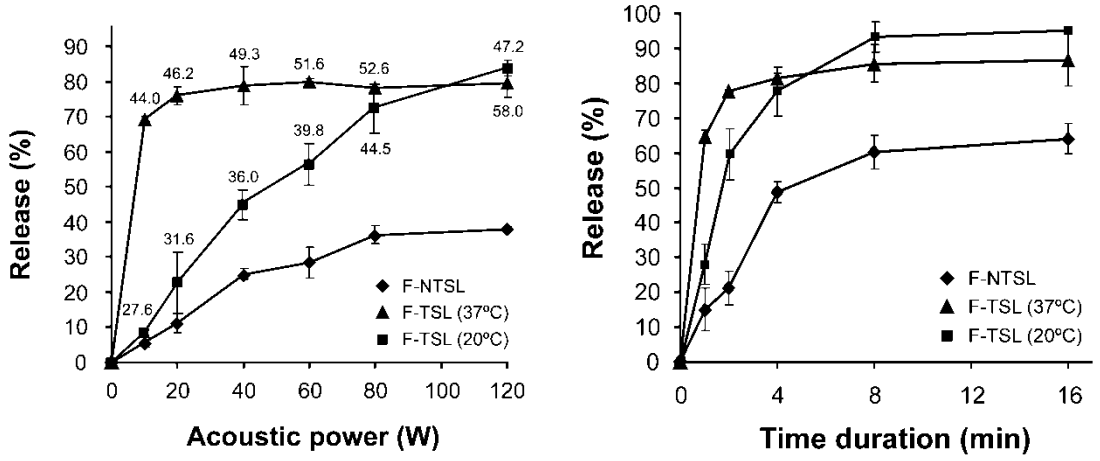


Fig. 2: HIFU-mediated release of fluorescein from F-NTSL and F-TSL (initial temperature of the liposome dispersions 20 and 37ºC, respectively) at different acoustic powers for 4 min (including sample temperatures for F-TSL) (a) and for different time durations at 80 W of acoustic power (b) (mean ± SD, n=3).

NR-TSL and NR-NTSL

Fig. 3 shows the release percentage of Nile Red from NR-TSL and NR-NTSL after exposure to HIFU. NR-TSL shows most release of Nile Red after 4 minutes of exposure to 120 W (48%). When exposed to 80 W at different time durations, most release was observed after 16 minutes (66%). Since lipophilic compounds are not released from liposomes through only temperature elevation and because no significant changes of liposomal size or PDI were observed, it can be concluded that HIFU reversibly destabilizes the lipid bi-layer. This has lead to the release of Nile Red.
Release of Nile Red from NR-NTSL resulted in higher release percentages. Up to 64% was released after exposure to 120 W for 4 minutes and up to 78% release was seen after exposure to 80 W for 16 minutes. The difference in release between the different types of liposomes can be explained by the lipid composition of both liposomes. NR-TSL consists mainly of DPPC (67% of the total composition), while no DPPC is present in NR-NTSL. NR-NTSL consists mainly of DSPC.
It has been found that liposomes containing paclitaxel (also a lipophilic compound) show less stability of the phospholipid bi-layer when DSPC is mainly present as compared to when DPPC is the main component.[5] This explains the higher Nile Red retention in NR-NTSL.

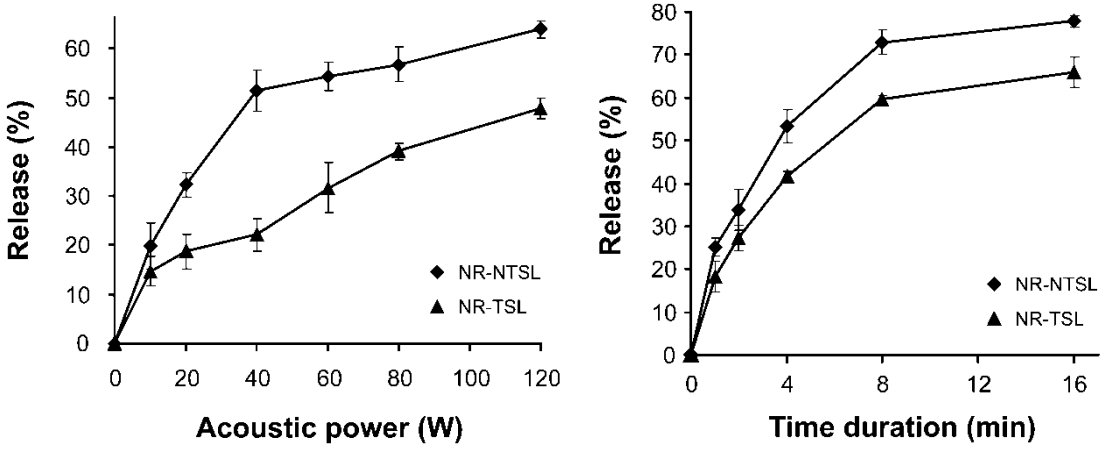


Fig. 3: HIFU-mediated release of Nile Red at different acoustic powers for 4 min (a) and for different time durations at 80 W of acoustic power (b), mean ± SD, n=3.

Pulsed HIFU-triggered release

Release of both hydrophilic and lipophilic compounds from liposomes is possible with HIFU. This release is not dependent on temperature. Continuous wave HIFU is accompanied by the generation of heat. Nowadays this technique is used for non-invasive thermal tumour ablation.[9] Pulsed HIFU is characterized by short ultrasound pulses with repeating duty cycles. This should prevent heat generation and is therefore interesting for this research.
The release of the dyes after exposure to pulsed-HIFU of up to 32 minutes at 80 W with a duty cycle of 10% is shown in Fig. 4. The release profile of fluorescein from F-NTSL is in line with the results as seen with continuous wave HIFU, taken into account that the time duration with pulsed-HIFU has to be divided by 10 due to the duty cycle of 10%. F-TSL showed a significantly higher release than F-NTSL. This can be explained by the lipid composition of the liposomes (see above).
No temperature elevation was observed with pulsed-HIFU exposure. This confirms the presence of an alternate release mechanism apart from temperature elevation.
Nile Red release from NR-TSL is comparable with the results from the continuous wave HIFU results. This is in line with the results from the fluorescein liposomes. However, the release from NR-NTSL after exposure to pulsed HIFU is significantly higher than was seen after continuous wave exposure. This suggests that pulsed HIFU is a more efficient way to trigger release of lipophilic compounds from liposomes as opposed to continuous wave HIFU.

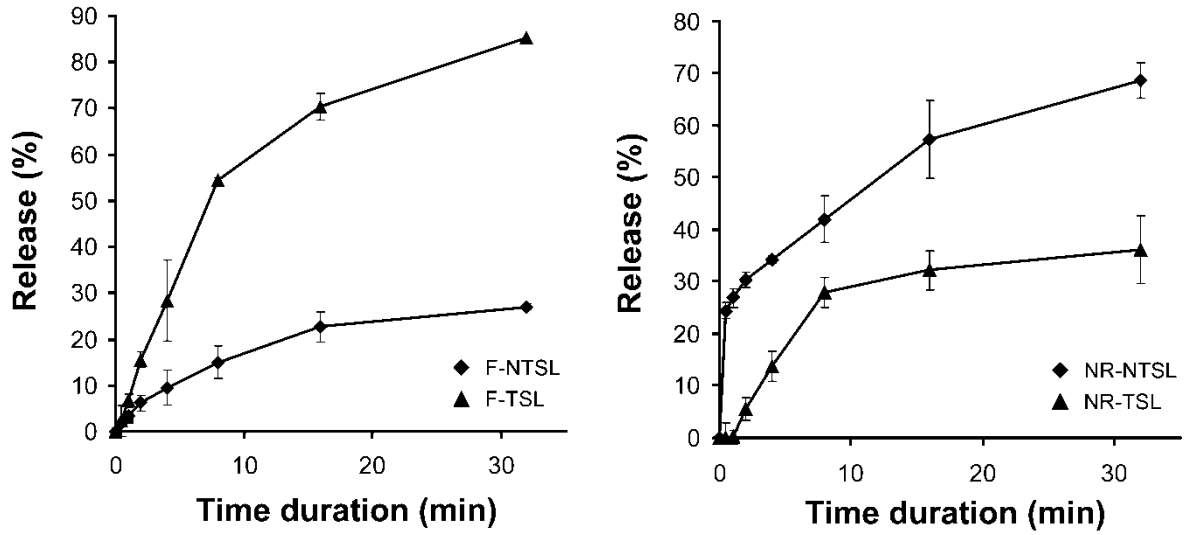


Fig. 4: Fluorescein release from F-TSL and F-NTSL (a) and Nile Red release from NR-TSL and NR-NTSL (b) after exposure to 80 W of pulsed-HIFU (duty cycle 10%) for different time durations, mean ± SD, n=3.

Pulsed HIFU-triggered release combined with microbubbles

In order to investigate whether the release of both fluorescein and Nile Red could be increased and accelerated, microbubbles were added to the liposome dispersions which led to the most release (F-TSL and NR-NTSL) and exposed to 80 W of pulsed-HIFU (duty cycle 10%) up to 8 min. As can be seen in Fig. 5, the release was decreased. Therefore it can be concluded that collapse of the microbubbles, called cavitation, is not the main mechanism behind the release of these compounds. It is hypothesized that the microbubbles absorb the energy of the HIFU instead of amplifying it. This, however, needs further research since there is no more proof for this statement than the reduced release seen in Fig. 5.

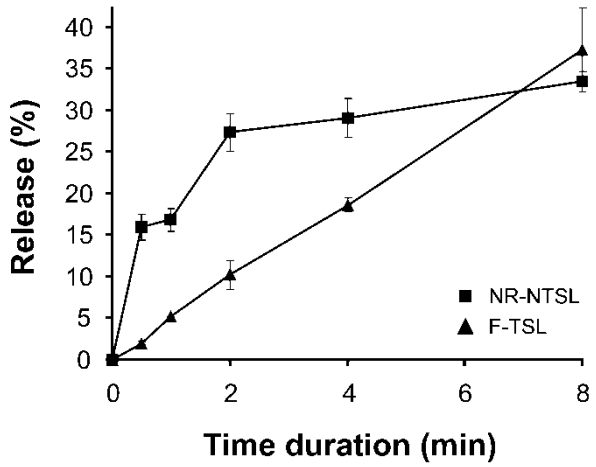
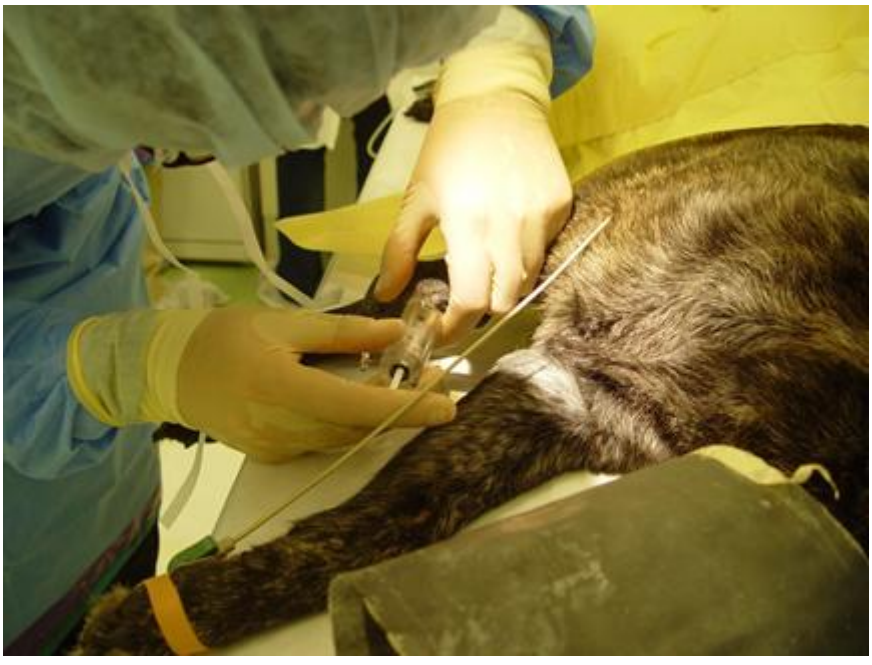


Fig. 5: Fluorescein and Nile Red release from F-TSL and NR-NTSL, respectively, after exposure to 80 W of pulsed-HIFU (duty cycle 10%) for different time durations, mean ± SD, n=3.

Conclusion

In conclusion, this study has demonstrated release of a hydrophilic compound from liposomes. Furthermore, extensive release of a lipophilic compound upon HIFU-mediated ultrasound-triggered release was shown. This study shows that it will be possible to regulate both hydrophilic and lipophilic drug-mimicking compounds from liposomes in a controlled manner by regulating the acoustic power and exposure duration. The exact mechanism of this release requires further study. However, it has to be taken into account that this study was performed in well-controlled environments. This study merely demonstrates the proof of concept that both hydrophilic and lipophilic compounds can be released from liposomes using HIFU. Further *in vivo* research should be done to determine actual possibilities for human and veterinary medicine.
This study does, however, show great promise for future drug delivery systems.

Holmium Therapy



Introduction

Due to advances in the healthcare system, humans and animals are able to become older than ever before. Unfortunately, high age is a high contributing factor for the development of cancer.[10]
Cancer is not a disease restricted to humans. Animals suffer from it as well. About 30% of dogs and cats will develop a form of cancer in their lifetime.[10]
Because of the increase in cancer, the medical world has developed several techniques to treat cancer. Surgical removal, chemotherapy, immunotherapy and radiotherapy are the most common therapies used. However, most of these therapies have some very serious systemic side effects.
This is one of the reasons research should be conducted towards local therapies. The Utrecht University Clinic for Companion Animals is collaborating with the Department of Nuclear Medicine of the University Medical Centre Utrecht to investigate and develop a new and promising therapy which would ensure a high local dose and very few side effects.
For a while now, patients with liver malignancies have been treated using microspheres loaded with the high-energy beta-emitting radio-isotope yttrium-90 (^{90}Y). By injecting these microspheres into the arteria hepatica, an embolization forms strictly within the tumours which are then irradiated. Since liver tumours are exclusively dependent on arterial blood supply this method will mainly target the malignancies and not the healthy tissue. High response rates have been reported. The only problem is that post-administration visualization of the ^{90}Y microspheres' biodistribution is incredibly difficult because of ^{90}Y's lack of gamma emission.[11]
This is the main advantage of poly(L-lactic acid) microspheres loaded with the radioisotope holmium-166 (^{166}Ho-PLLA-MS). Apart from high-energy beta particles, ^{166}Ho also emits low-energy gamma rays. These gamma rays allow for nuclear imaging of a sufficient quality for quantative assessment. It is also a highly paramagnetic element which allows for Magnetic Resonance Imaging as well.[11]
The high-energy beta particles emitted by ^{166}Ho have a maximum range of 8.6 mm in soft tissue. This allows for very accurate irradiation. Furthermore, ^{166}Ho has a favourable short physical half-life of 26.8 hours.[12]

The Utrecht University Clinic for Companion Animals has been exploring the use of ^{166}Ho-PLLA-Microspheres for use as a treatment in solid tumours in veterinary patients. The results gathered from these patients show great variation and the outcome of the therapy is very hard to predict. This is due to the inconsistency in the types, sizes and consistencies of the treated tumours. To be able to give an accurate prediction it is vital that more data is gathered from different tumours.
The gathering of more data will be the goal of this internship and this particular study.

Tara

Pre-treatment

On the 16th of May, patient Tara Aarts (1205017) was presented at the University Utrecht Clinic for Companion Animals for a thickness under the tongue.
On the same day, a CT-scan was made to see whether any metastasis were present. This was not the case. Furthermore a thin needle aspiration biopsy was taken from the tumour. The result indicated that the tumour was a badly differentiated squamous cell carcinoma. The tumour was located on the frenulum of the tongue.
During the physical examination we found that there was nothing wrong with the cat. It was very docile and did not appear to be experiencing any trouble caused by the tumour.
When the cat was being sedated however, the docility was gone and the patient became highly aggressive. This was to be taken into account when we would decide whether or not Tara was suitable for Holmium therapy. The owner also told us that the aggressive behaviour was well known to her and that strangers feeding her has been a problem in the past.
Since the tumour was located on the base of the tongue, Tara would have to fed by a stomach tube to ensure a proper feeding regime. This would prove quite difficult concerning the aggressiveness towards strangers.
All in all, however we did decide that Tara was a suitable patient for Holmium therapy since she had no metastasis, her blood values were good, the tumour was inoperable and the owner was motivated. We would be able to handle the aggressiveness well enough.
The Holmium treatment was scheduled for the 5th of June.

Unfortunately, I received a troubling mail on the 20th of May. The owner told me that Tara was doing great after the visit to the clinic. But on the evening of the 19th of May Tara started to get some diarrhoea. The next evening she experienced some more diarrhoea after the administration of some Metacam. The owner was quite distraught and wondered whether the pain of Tara was worth the trouble of waiting for the Holmium therapy. She was strongly considering euthanasia. After explaining quite thoroughly, by myself and Prof. Dr. Kirpensteijn, that diarrhoea is a known side effect of long term Metacam administration and that there was nothing to worry about, the owner was calmed down enough to let Tara live.

The next week, Tara was doing slightly better. The diarrhoea had stopped but it was apparent that eating and drinking was getting more and more difficult. This is why we scheduled a preliminary laser resection of the tumour on the 31st of May. The result can be seen in Fig. 6.

Since everything appeared to be going according to schedule, the forms to request the holmium microspheres were completed and given to Remmert de Roos (see attachment 1).

When the patient arrived on the 31st of May it was clear that the tumour had increased in size. The patient however did not appear to be suffering clinically. After the patient was sedated, she was transported to the operation room.
Unfortunately, the laser was broken so we had to resort to another method of tumour resection. A large portion of the tumour was removed using the Ligasure. An ellipsoid tumour of 2x2x0.5 cm was left to be treated with radioactive holmium microspheres.
The operation was completed successfully and the patient woke up without any trouble.

On the 3rd of June however we received an email from the owner that the patient had unfortunately passed away. The patient had some trouble eating and drinking the day following the operation,

which is perfectly explicable concerning the location of the operation wound. It was still clear that the drive to eat was present.
The next day however, the will to eat and drink appeared to be gone. Early in the day, the patient fainted and the owner found her lying in a puddle of blood. After bringing the patient to the local vet, the wound (location unclear to us. Apparently not the operation wound) was closed and the patient died during the narcosis due to a loss of too much blood.
As soon as possible, the owner was contacted to show our support and compassion. A very unfortunate and unexpected turn of events.

Fig. 6: Tumour after cauterization resection.

Pre-treatment

On the 25th of April, Farkas Leeh (1203928) was presented to the University Utrecht Clinic for Companion Animals with a thickness on the left thigh. At the end of October 2011 the thickness was diagnosed as a cyst and the veterinarian advised the owners to do nothing about it and come back in a month to see if anything had changed.
On the 17th of April the owners returned to their veterinarian and the cyst had grown tremendously. The veterinarian was unable to empty it of fluid completely and raised a suspicion of different compartments. However, it was possible to drain around 200 ml of clear, yellow, foaming and fibrinous fluid from the thickness and the tension was relieved. The thickness still remained and a hard mass was felt inside. Concerning the size of the mass, Farkas was sent to the University Utrecht Clinic for Companion Animals.
On the 25th of April, an echo was made and a biopsy of the mass was taken. The biopsy showed that the mass was a hemangiopericytoma. A picture of the mass can be seen in Fig. 7. The echo results are shown in Fig. 8.

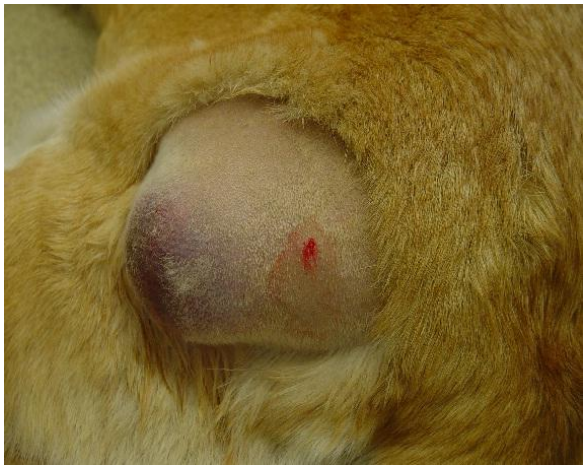


Fig. 7: Mass as presented on the 25th of April.

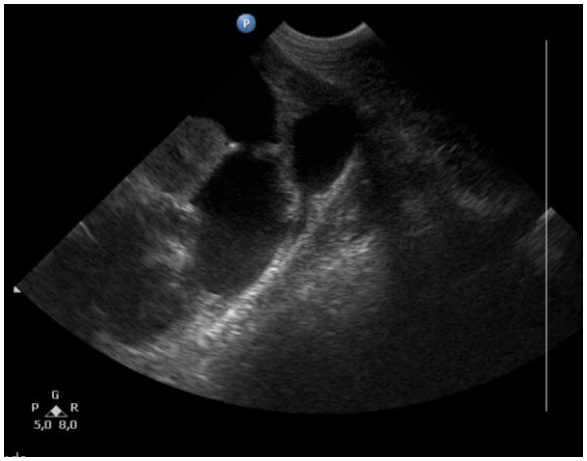


Fig. 8: The echo of the tumour shows a cystic aspect with several components

On the 1st of May, Farkas was back at the clinic once more for a CT-scan (Fig. 9). The scan pointed out that the thickness was mostly filled with fluid. Furthermore, the scan showed a fairly round tumour with measurements of 10x11x13 cm. The tumour itself however, was hardly palpable because the skin covering the tumour was too tense.
Although closely related, the mass showed no connections with the underlying m. Biceps femoralis. Furthermore, no lymph nodes appeared to be affected in the area of the leg.
Other findings were that Farkas had slight indications of a hernia at the lumbosacral passage, no anomalies in the lungs except for some atelactasis, spondylosis in the thorax and some lipomas.
It was concluded that the mass showed no invasion in the surrounding tissues except for some linear structures, which might be vessels, on the craniodistal side. Furthermore there are no indications of metastasis in the local lymph nodes or the lungs.
The entire mass was measured to be 10x11x13 cm. Because of the extensive amount of fluid it was hard to estimate what part of the mass was exactly soft

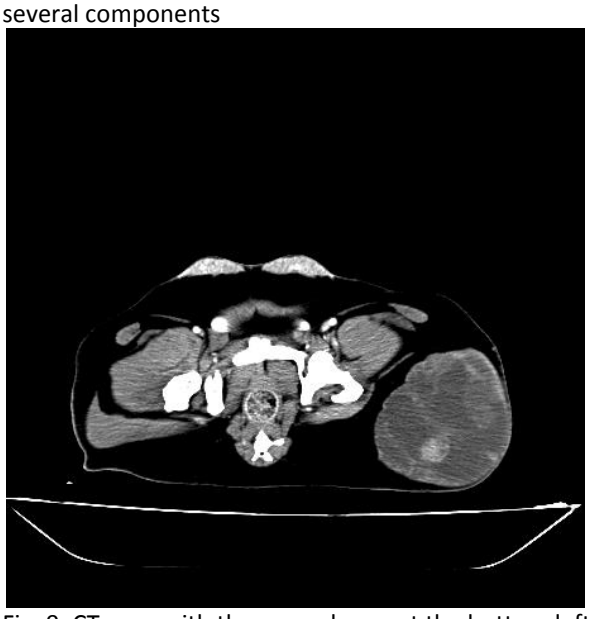


Fig. 9: CT-scan with the mass shown at the bottom left.

tissue tumour.

The owners were informed that there were two therapeutic options. Firstly, a marginal resection followed by irradiation. Secondly, holmium irradiation followed by marginal resection. The owners were informed that our preference would be holmium irradiation so that the tumour would be reduced in size and the surgical margins would be smaller. Luckily they decided that this was the treatment they wanted as well. So it was decided that Farkas would be treated with holmium microspheres on the 5th of June.
Two weeks before the treatment would commence, Mr. Leeh informed us that the skin covering the mass was under great tension and that an ulcer had started to form (Fig. 10). At night they protected the wound and mass using a contraption of an old T-shirt and suspenders (Fig. 11).
To prevent wound infection, we advised the owners to cover the ulcer with some sterile bandage gauze (Fig. 12).

On the 29th of May, the request form (att. 2) for the Holmium microspheres was sent to Remmert de Roos.

Since the tumour produced a large amount of liquid at a rapid rate, Farkas was back on the 30th of May to empty the tumour and to do some last checkups. The mass had grown in size to 20x25x9 cm (estimated) and the tension on the skin had greatly increased.
Under sedation, the tumour was drained of about 500 ml of fluid. Furthermore, venous blood was gathered to check whether Farkas was healthy enough to receive the holmium therapy. Luckily, no anomalies were discovered in the blood values except for a slightly lowered albumin. This was not enough to be concerned about. Since Farkas passed all the exclusion criteria for the holmium therapy, she was now ready to be treated a week later.

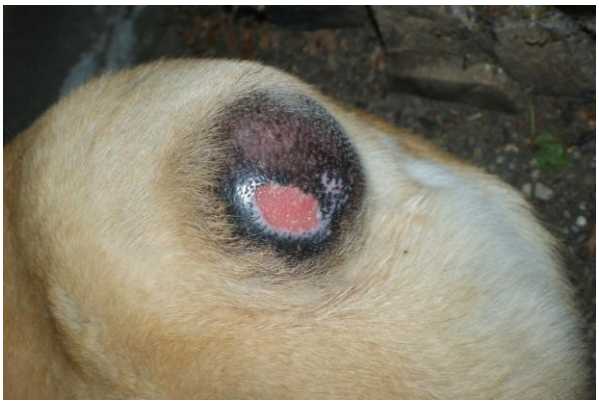


Fig. 10: Mass with ulceration. Photographed on 23th of May.

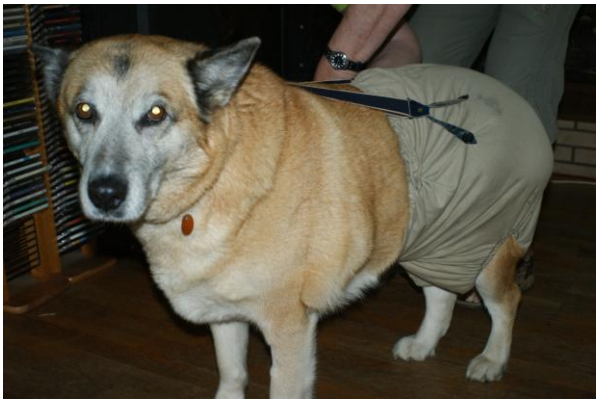


Fig. 11: Farkas with the T-shirt contraption to prevent licking.

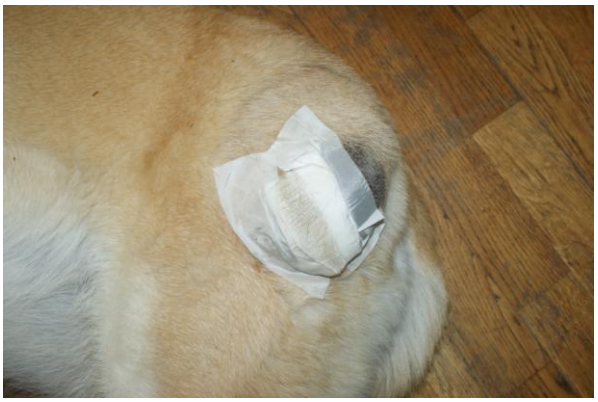


Fig. 12: Mass covered with sterile bandage gauze to prevent infection of the ulcer.

Treatment

On the 5th of June, Farkas was treated with ^{166}Ho-PLLA-Microspheres. Because of the size of the tumour, the 2500 Mbq with a dose of 200 Gy was injected. This was the maximum dose. The procedure was completed without any complications.
Afterwards, Farkas woke up feeling a bit confused and dizzy but otherwise fine (Fig. 13 and Fig. 14).

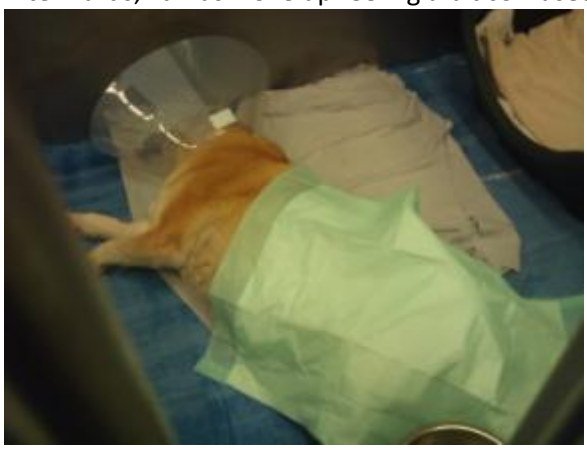


Fig. 13: Farkas shortly after the injection of the holmium microspheres.

Fig. 14: Farkas awake after the injections.

Hospitalization

Because of the radiation emission of the tumour, Farkas had to be hospitalized until the emission was deemed safe. Every day, except for in the weekend, I took care of Farkas at 8:00, 12:00 and 16:00. Furthermore, I called the owners everyday to talk to them about the condition of Farkas.
The hospitalization period passed without many incidents. The only incident that occurred was on the second night when no one came to take care of Farkas. The following day she was very eager for company, water and food. Furthermore, she had broken her collar in the night. Luckily, it did not appear that she had started licking the injection sites. During the entire period, the faeces had a good aspect and Farkas appeared very healthy, enthusiastic and hungry dog. This enthusiastic nature resulted in the destruction of three collars, which were replaced in time before any damage to the tumour was done.
Each day of the hospitalization, the radiation emission from the tumour was measured. The data concerning these measurements is presented in the attachments (att. 3).
On the 11th of June, the radiation was deemed to be safe enough for Farkas to be retrieved by her owners. So on the 12th of June, Farkas was greeted by her very eager owners.

Fig. 15: Farkas outside during hospitalization.

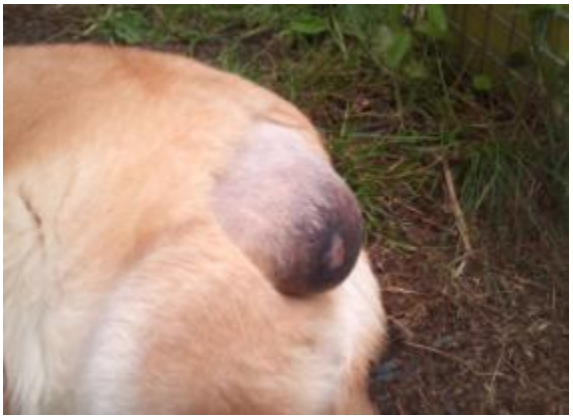


Fig. 16: The tumour on 11-06. One day before release.

Follow up

During the following period of time Farkas returned to the University Utrecht Clinic for Companion Animals.

27th of June

Farkas was very lively and playful. She ate well but seemed to drink a bit less than before. However, the faeces and urine didn't seem to be abnormal. Farkas had gained three kilos over the past few weeks but this was because of a different food during and after the hospitalization.
The general impression of Farkas was that of an alert dog.
The breathing frequency was hard to judge because she was panting. It was costo-abdominal.
The pulse was hard to feel because of the excess fat. Heart auscultation gave us a frequency of 46 beats/minute. Furthermore, no heart murmurs were heard.
The temperature of Farkas was 38.7 °C. Her mucous membranes were all pink. The sclera was white and the CRT was less than one second.

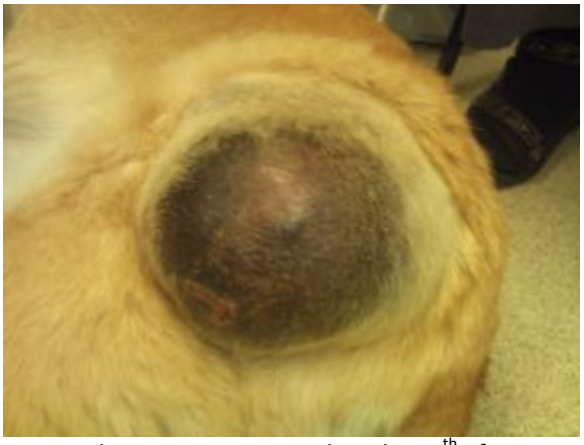


Fig. 17: The mass as presented on the 27th of June

Her lymph nodules, skin, hair and horny structures also showed no abnormalities.
Overall Farkas appeared to be doing very well and not to be suffering from any side effects of the Holmium treatment.
The thickness did not appear to be any bigger than when Farkas left the clinic two weeks before (Fig. 17). According to the owner she has not touched the thickness herself. At home she wears the collar all the time.
The thickness was soft and Farkas barely reacted to touching of it. Fluid appears to be the main component of the thickness. It was measured to be 11x13x15 cm (depth was estimated).

11th July

Farkas was still very playful. She ate, drank, urinated and defecated very well. The general impression was that of a lively dog, who is a bit too fat, but appears to experience no troubles from the mass on the hip.
The breathing was still hard to judge due to panting. It was costo-abdominal.
We were able to judge the pulse this time. It had a frequency of 120 beats/minute, was powerful, regular, equal and symmetrical.
The temperature was 38.3 °C. No abnormalities were found in the mucal membranes, lymph nodules, skin, hair and horny structures.
It still appears that Farkas has no troubles with any side-effects from the Holmium treatment.
The thickness still had a soft consistency (Fig. 18). It was warm to the touch but Farkas did not appear to be troubled by us touching the mass. The size of the mass was 15x15x12 cm (depth was estimated).

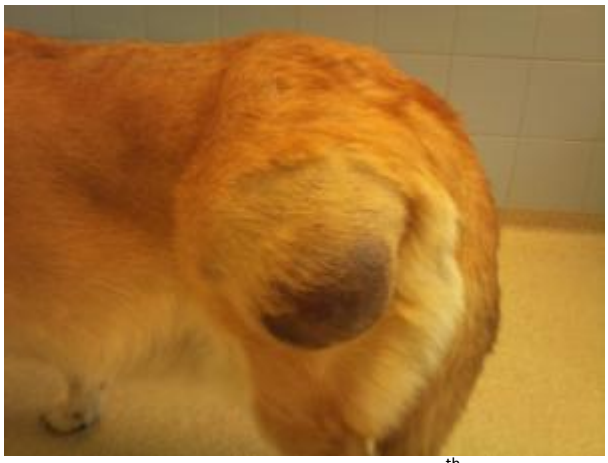


Fig. 18: The mass as presented on the 11th of July

In consultation with the owners it was decided that Farkas would be scheduled for her operation on the next day. Prior to the operation an CT scan would be made to clearly see the margins of the tumour and decide the operation method.

12th July

Prior to the operation. A CT scan was made of the mass and of the thorax. The CT scan shows a clear deduction of soft tissue in the tumour (Fig. 19). Due to the high fluid content of the mass, it is difficult to judge the exact difference in soft tissue from the CT scan made before the Holmium treatment.

On the thorax scan, two nodules can be seen in the lungs (Fig. 20). These were not there on the earlier CT-scan and might indicate that the tumour has spread to the lungs.

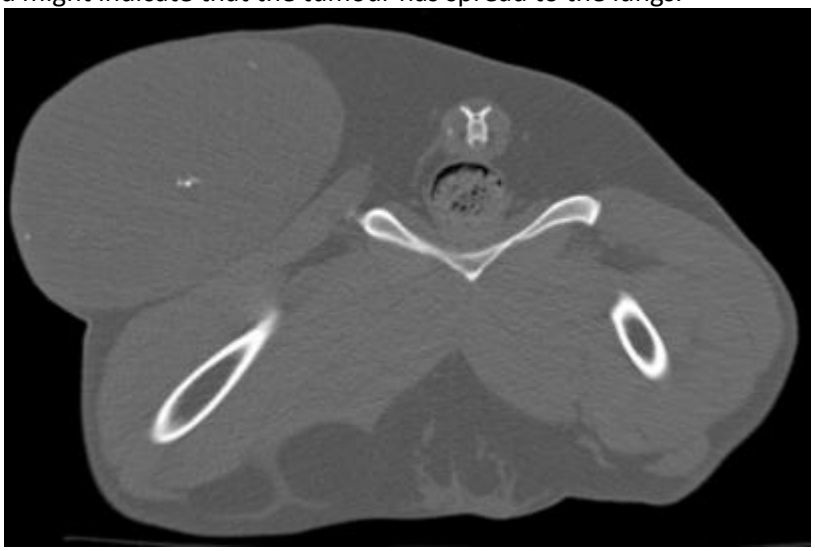


Fig. 19: CT scan of the mass on 12-07

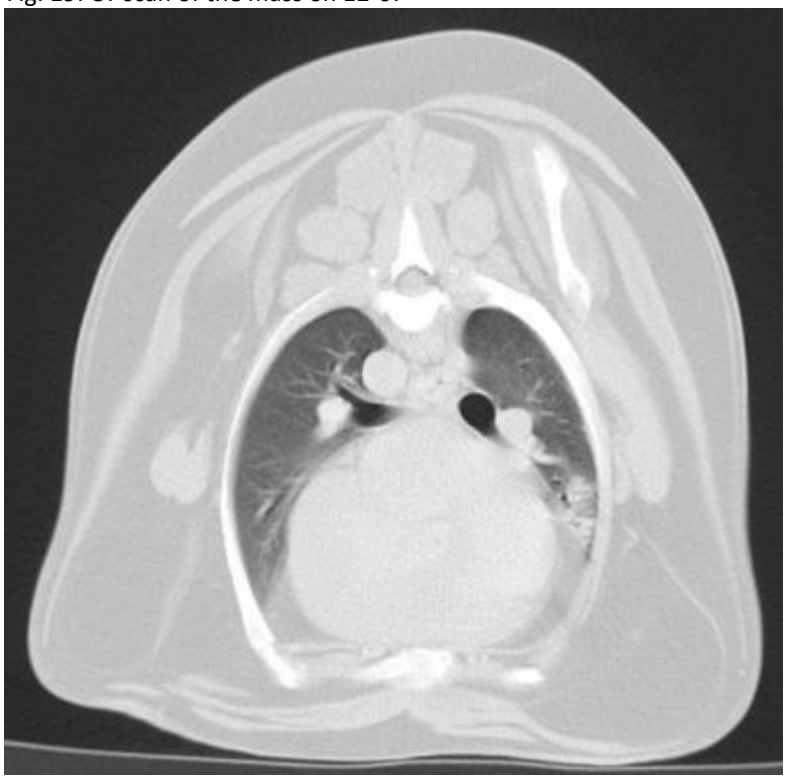


Fig. 20: CT scan of the thorax on 12-07

After a quick reconsideration it was decided to remove the tumour anyway. During the operation, the owners were informed of the bad news and of the following steps concerning the possible metastasis. Meanwhile, Farkas was being prepared for surgery (Fig. 21).

The operation was started by marking the cutting margins. About 1.5 centimetres away from where the skin was clearly attached to the tumour, a line was drawn. Thus, a circle with a diameter of about 15 centimetres was drawn around the tumour (Fig 22).
The incisions were made so that as much skin as possible was preserved. The tumour was separated from the skin and deeper tissues manually, a pair of scissors and a monopolar cauterizer (Fig. 23-25).
During the operation the tumour was damaged and it started to leak some fluid. The fluid was a clear, watery liquid.

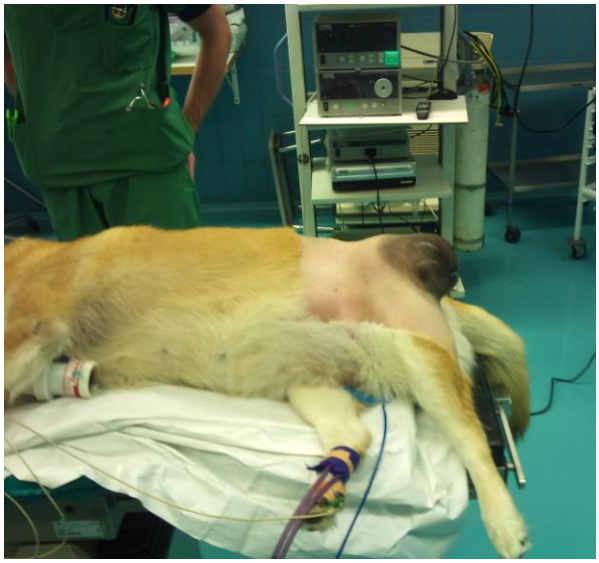


Fig. 21: Farkas before the surgical resection of the tumour.

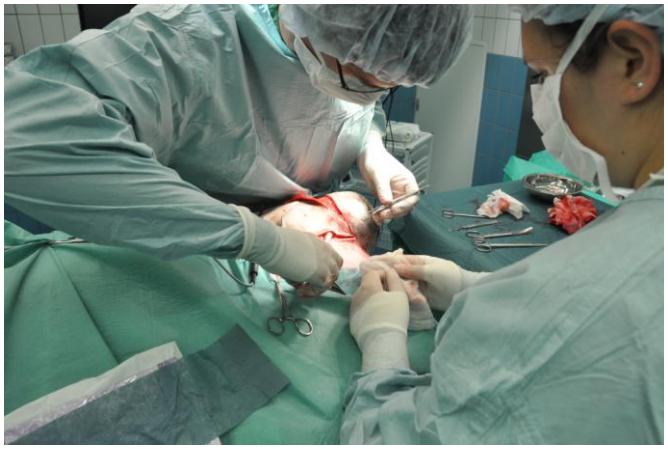


Fig.24: Resection using a pair of scissors.

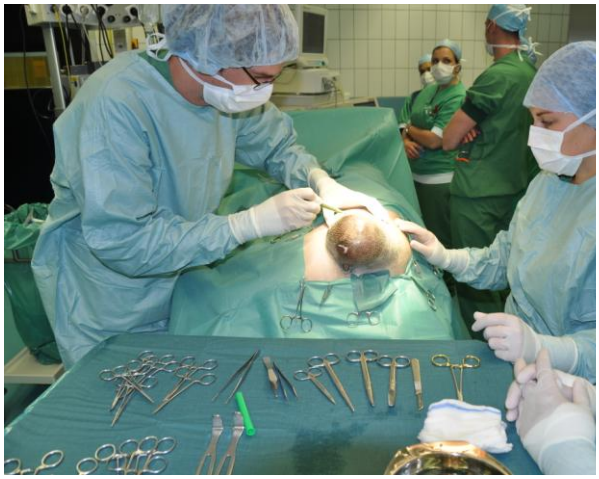


Fig. 22: Marking the tumour margings for resection.

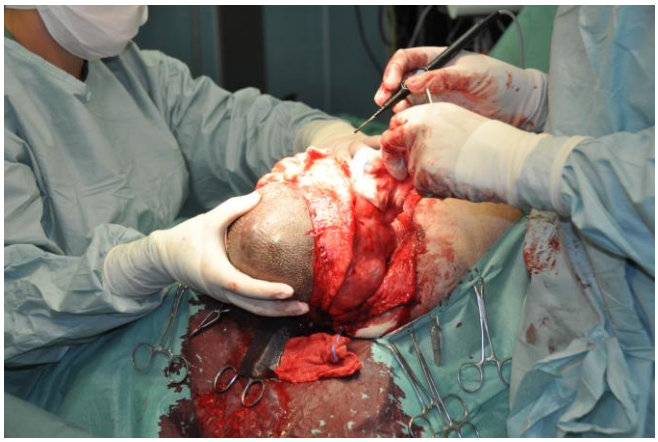


Fig. 25: Resection of the tumour shortly before removal.

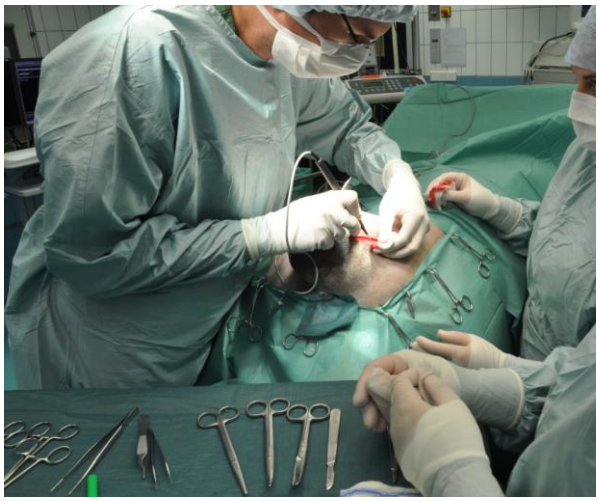


Fig. 23: Resection using the cautherizer.

When the tumour was removed (Fig. 26), the resulting wound (Fig. 27) was washed with warm NaCl solution and the instruments and gloves were changed.
The wound was too large to be closed using simple sutures. Hence, a transposition flap was made. Furthermore, walking sutures were used to bring the wound edges closer together. The skin was closed using several simple sutures (Fig. 28).
Since the wound produced a lot of fluid during the closure and a cavity had formed, a small suction drain was placed in the cavity.

The mass was sent to the pathologist. The results are added in the attachments section (att. 4). Fig. 29 shows the microscopic image of some holmium microspheres inside the tumour. Fig. 30 shows the macroscopic aspect of the tumour.

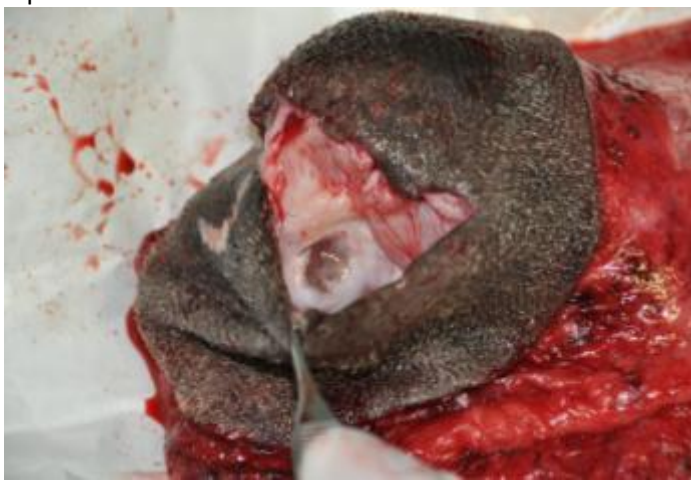


Fig. 30: Macroscopic aspect of the tumour after incision

25th July

After the operation an appointment was scheduled to check the wound and possibly to remove the stitches.
The owners told us that immediately after coming home the day after the operation, Farkas was very active and playful. She ate and drank well.
The wound had just opened in the car and thus showed an opening of 5-6 cm located on the place where the tension was highest on the stitches. There was some necrotic skin around the stitches.
The owner told us that two days after surgery the wound excreted a lot of fluid which was alternately bloody and clear. However, no pus came out.
The general condition of Farkas was great. No abnormalities were found except for the left lnn. Accessorius which appeared to be slightly enlarged.
The stitches were not removed as of yet. The necrotic skin would fall of eventually and this wouldn't be a problem. If the wound would get dirty, the owner would need to clean it with some water.

Fig. 26: The tumour.

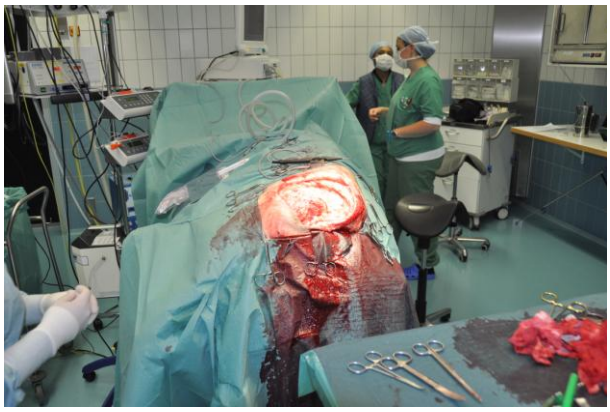


Fig. 27: The resulting wound.

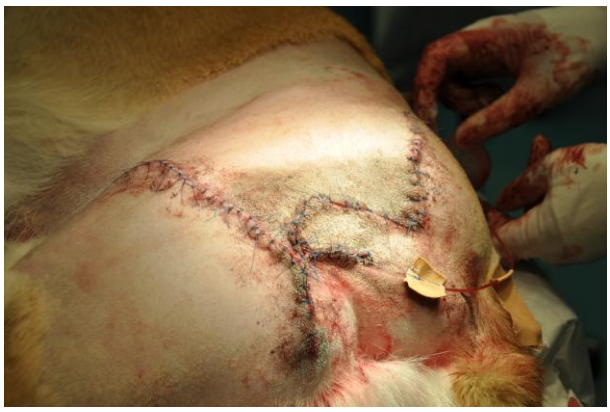


Fig. 28: The resulting wound after closure.

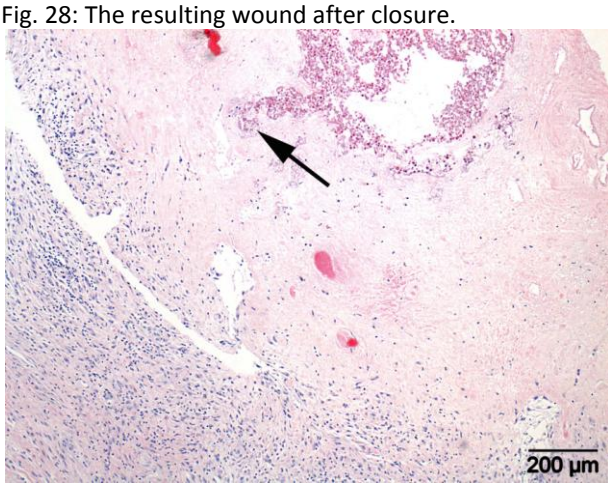


Fig. 29: Microscopic image showing the Holmium microspheres in the tumour (arrow).

This was the last time I saw Farkas. I know she is still doing great today because I am still in contact with the owner. Fig. 31 and 32 show the last pictures of the wound I received. They were taken on the 30th of July.

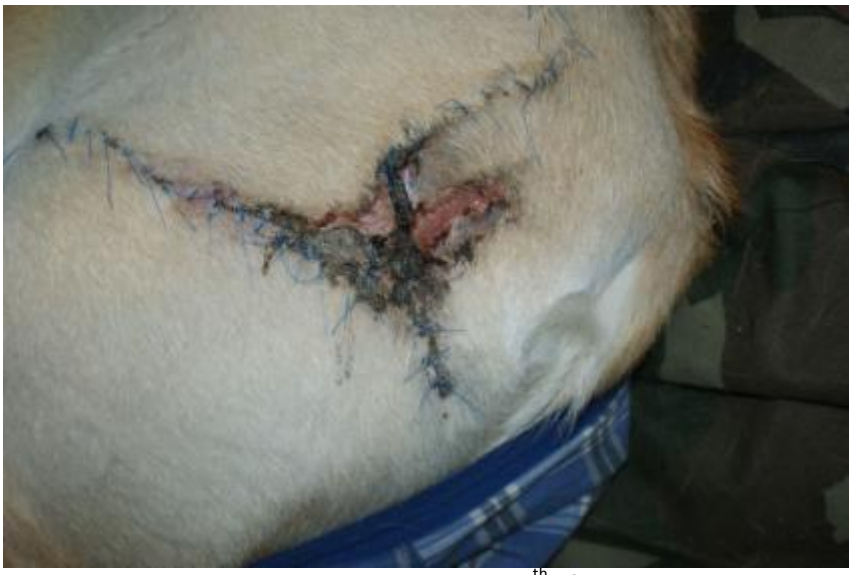


Fig. 31: The wound on the 30th of July.

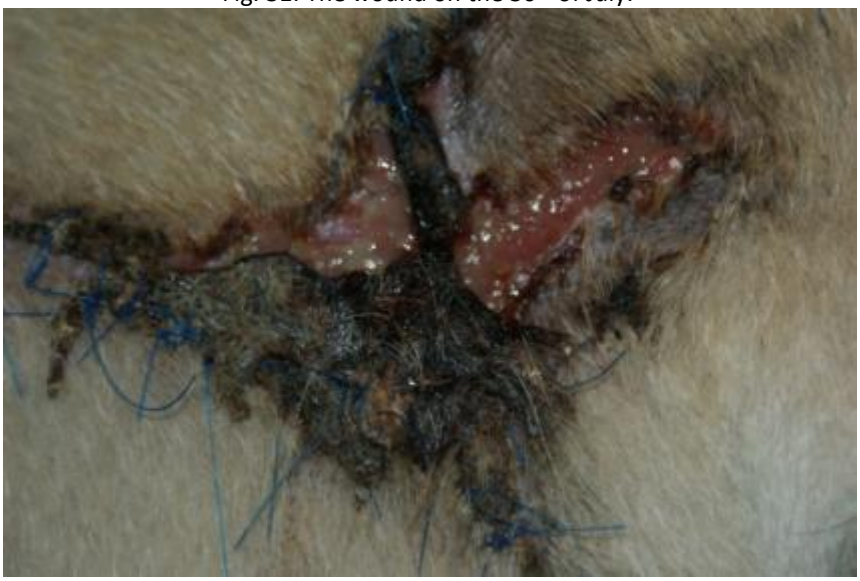


Fig. 32: A close-up of the necrotic tissue on the wound.

Attachments

Attachment 1

Aanvraagformulier radionuclidenapotheek

Datum aanvraag	29-05-2012
Akkoordverklaring getekend	**Ja** / Nee
Naam coassistent	Willem van Doesum

Patiënt

Naam patiënt	Tara
Patiëntnummer	1205017
Diersoort	Kat
Ras	Huiskat
Geslacht	Vrouwelijk
Gewicht	5.8
Geboortedatum	19-4-1997
Naam eigenaar	Karin Aarts
DA UKG	Prof. Dr. Jolle Kirpensteijn
Onderzoeker(s) UMC	Frank Nijsen

Tumor

Type tumor	Plaveiselcelcarcinoom
Locatie tumor	Frenulum tong
Afmetingen tumor ☐ CT, ☐ lineaal	2 x 2 x 0.5 cm (na laserresectie)
Tumorvolume	$V=4/3*pi*(0.5*(d1+d2+d3))^3$ Geeft $V = 0.52\ cm^3$
Methode volume berekening	***Ellipsoïde**
Consistentie tumor	**Zacht** / Matig / Hard
Foto's/DB doorgestuurd naar UMC	Ja / Nee

Behandeling

Studieprotocol	PCC kat
Dosiscohort (Gy)	200 / 400 / **600** / 800
Datum behandeling	05-06-2012
Radioactieve stof	166-Ho
Aangevraagde hoeveelheid activiteit	Op tijdstip toediening (zie protocol): 35,6 MBq
Hoeveelheid microsferen (mg)	200 mg
Gewenste aankomstdatum UMC	05-06-2012
Gewenste aankomsttijd UMC	10:00
Gewenste aankomsttijd DGK	12:00
Tijdstip toediening	

*: Volgens onderzoek Dorien
Methode 2: het volume van tumoren meten in CT-beelden; uitgaan van ellipsoïde
Methode 3: het volume van tumoren meten in CT-beelden; mbv Impax, uitgaan van cilinders
Methode 4: het volume van tumoren meten in CT-beelden; mbh van ImageJ

Verzorging

IV cathether in welke poot?	LV/RV/LA/RA
Antibiotica Convenia dosis:	SC 0,58 ml
Tramadol dosis:	nvt
Meloxican dosis:	Afhankelijk van bijwerkingen..
Temgesic dosis:	20 ug/kg 4dd SC
Fetanyl patch	nee
Andere medicatie	
Sonde voeding ja/nee: hoeveelheid:	nvt
Verzorgingsrooster ingevuld:	Ja/nee
DA UKG tel nummers	06-41434564 JK 06-45782076 BvN (vanaf 18-06-12)

Attachment 2:

Aanvraagformulier radionuclidenapotheek

Datum aanvraag	29-05-2012
Akkoordverklaring getekend	**Ja** / Nee
Naam coassistent	Willem van Doesum

Patiënt

Naam patiënt	Farkas
Patiëntnummer	1203928
Diersoort	Hond
Ras	Kruising
Geslacht	Vrouwelijk
Gewicht	42.2 kg
Geboortedatum	01-05-2003
Naam eigenaar	Marten Leeh
DA UKG	Prof. Dr. Jolle Kirpensteijn
Onderzoeker(s) UMC	Frank Nijsen

Tumor

Type tumor	Hemangiopericytoom
Locatie tumor	Linkerheup
Afmetingen tumor ☐ CT, ☐ lineaal	8 x 8 x 8 cm
Tumorvolume	$V=4/3*pi*(0.5*(d1+d2+d3))^3$ $V=1071{,}8\ cm^3$
Methode volume berekening	***Ellipsoïde**
Consistentie tumor	Zacht / **Matig** / Hard
Foto's/DB doorgestuurd naar UMC	**Ja** / Nee

Behandeling

Studieprotocol	hemangiopericytoom
Dosiscohort	**200** / 400 / 600 / 800 (ong 100 Gy)
Datum behandeling	05-06-2012
Radioactieve stof	166-Ho
Aangevraagde hoeveelheid activiteit	2.5 GBq (bij binnenkomst UKG)
Hoeveelheid microsferen (mg)	400 mg
Gewenste aankomstdatum UMC	05-06-2012
Gewenste aankomsttijd UMC	10:00
Gewenste aankomsttijd DGK	12:00
Tijdstip toediening	

*: Volgens onderzoek Dorien
Methode 2: het volume van tumoren meten in CT-beelden; uitgaan van ellipsoïde
Methode 3: het volume van tumoren meten in CT-beelden; mbv Impax, uitgaan van cilinders
Methode 4: het volume van tumoren meten in CT-beelden; mbh van ImageJ

Verzorging

IV cathether in welke poot?	**LV/RV**/LA/RA
Antibiotica Convenia dosis:	SC 4.2 ml
Tramadol dosis:	nvt
Meloxicam dosis:	nvt
Temgesic dosis:	20 ug/kg 4dd SC
Fetanyl patch	Ja/**nee**
Andere medicatie	Carprofen 2 mg/kg 2dd PO
Sonde voeding ja/nee: hoeveelheid:	nvt
Verzorgingsrooster ingevuld:	Ja/nee
DA UKG tel nummers	06-41434564 JK 06-45782076 BvN

Attachtment 3:

Stralingsmetingen holmium behandeling

^{166}Ho-AcAc-MS behandeling

Stralingsmetingen na ^{166}Ho-AcAc-MS behandeling

Instructies:
Vul dit werkboek s.v.p. in op de dagen na de behandeling.

Patiëntensticker

Datum behandeling (DD/MM/JJJJ):

05-06-2012

Datum	Tijd	Achtergrond-straling (µSv/h)	Straling op 10 cm (µSv/h)	Straling op 50 cm (µSv/h)	Straling op 100 cm (µSv/h)	Ontlasting (µSv/h)	Urine (µSv/h)
06-06	**12:00**		**750**	**22**	**9**	**-**	**-**
07-06	**15:00**	**0.13**	**300**	**12**	**3**	**-**	**-**
08-06	**14:00**	**0.15**	**160**	**6**	**1.6**	**-**	**-**
09-06	**8:00**	**0.10**	**30**	**9**	**1.9**	**-**	**-**
10-06	**08:30**	**0.15**	**16**	**6**	**0.7**	**0**	**-**
11-06	**12:00**	**0.14**	**45**	**0.7**	**0.16**	**<1**	**-**
12-06	**8:00**	**0.12**	**21**	**0.2**	**<**		

Datum + tijdstip ontslag patiënt:

12-06-2012 14:00 **uur**

Attachment 4:

Pathology results tumour Farkas

Langzaam groeiende dikte. Reeds bijna 20 cm doorsnede na een jaar. Op DNAB haemangiopericytoom. Dikte voor groot deel met vocht gevuld en duidelijk ook weefselmassa daarbij op CT. Is behandeld met radioactief holmium 6 weken geleden. Toen waren er geen aanwijzingen voor metastasen. Nu opnieuw CT van tumor om effect holmium te beoordelen en thorax. Nu 1 kleine noduli in long gezien, waarschijnlijk metastasen. Tumor marginaal verwijderd. Huid zoveel mogelijk gespaard voor wondsluiting dus dicht van de tumor afgeprepareerd. Op enkele huidlokatie waar macroscopisch twijfel over nabijheid tumor was zijn hechtingen geplaatst. De tumor is aan de huidzijde ingesneden om vocht af te laten lopen en tumormassa in onderliggende holte ook ingesneden voor betere fixatie. Klein stukje uit tumormassa in holte apart in potje (2). Lijkt lokatie van holmium te zijn maar is ook erg stevig (calcificatie?).

Macroscopie:
2 potten met weefsel.
Pot 1:
Verhouding formaline staat tot weefsel niet minimaal 10 staat tot 1. Pot opening te smal voor weefsel. Pot moeten openknippen om weefsel uit pot te halen.
Reeds ingesneden week aanvoelend met behaarde huid bedekt prominerend proces van ca. 11 tot 13 cm doorsnede. Subcutaan is een hoeveelheid weefsel weggenomen met afmetingen van ca. 19 x 13 cm. Aan 1 zijde van het weefsel waar zich een korst-achtig prominerend proces van ca. 1,5 x 0,5 cm bevindt zijn 4 hechtingen aanwezig; 2 net onder de huidlaag en 2 ca. 1 tot 2 cm daaronder. Aan tegenoverliggende zijde van het proces is net onder de huidlagen een derde en vijfde hechting aanwezig. Groter proces is over een lengte van ca. 8 cm reeds ingesneden. Verder gekliefd vanuit reeds aanwezige snede. Materiaal is zo goed als ongefixeerd. Materiaal moet nagefixeerd worden. Gekliefd uitgaand evan reeds aanwezige snede. Op snijvlak deels cysteus deel wit spekkig aspect. In cassettes 1 t/m 4 ingesloten excisies ter hoogte van hechtingen. In cassettes 5 t/m 7 ingesloten 4 excisies elder waar het proces marginaal verwijderd lijkt te zijn.
Pot 2 wit matig stevig onregelmatig weefsel van ca. 1 cm doorsnede. Gekliefd. Op snijvlak bruin goed omschreven aspect met een doorsnee van ca. 0,5 cm. Snijvlak en een snijrand ingesloten in cassette 8.

Microscopie:
Behaarde huid met in de dermis en subcutis een sterk wisselend celrijke mesenchymale neoplasie bestaand uit langgerekte spoelvormige cellen die deels in bundels zijn gelegen en op meerdere plaatsen concentrische whorling rondom vasculaire structuren vertonen. Daarnaast echter ook foci met concentrische whorling van de spoelcellige component rondom meer solide structuren. De neoplastische cellen bevatten gering in grootte variërende afgeronde tot ovale kernen met fijn reticulair chromatine, overwegend kleine nucleoli en een lage mitose-index. Tussen de tumorcellen een variabele hoeveelheid collagene matrix die deels zeer compact is afgezet maar deels ook een zeer losmazig aspect vertoont. Met name in de periferie multifocaal perivasculair gelegen lymfocytaire infiltraten.
In het separaat bijgeleverde biopt een vergelijkbaar histologisch beeld van de neoplasie met confluerende velden van necrose waarin zich grote aggregaten van afgeronde eosinofiele granulaire structuren bevinden (holmium microspheren). Op relatief beperkte afstand van de holmium microspheren bevindt zich vitaal ogend neoplastisch weefsel.

Conclusie:
Poot: laag maligne spoelcellig sarcoom met kenmerken van een hemangiopericytoom.
Lijkt (lokaal zeer krap) volledig verwijderd.

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