

University of Veterinary Medicine Hannover

**Evaluation of the cyclic combination chemotherapy protocol
including doxorubicin effects on cardiac parameters**

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I. Introduction

Doxorubicin is an anthracycline derived from the *Streptomyces* yeast. It has multiple mechanisms of action. These include intercalation of DNA, which leads to inhibition of protein synthesis and free radical formation, and inhibition of topoisomerase enzymes (LORI et al. 2010). DNA topoisomerases are the enzymes responsible for the conversion of DNA topology and are thus essential for many aspects of genetic processes (WANG 1985). By inhibiting DNA synthesis, DNA dependent RNA and protein synthesis are also inhibited. Main damage is produced by binding to the DNA (intercalation) and changing its three-dimensional structure. As a consequence, intercalation involves an increase in the vertical separation between adjacent base pairs and partial unwinding of the double helix, causing changes of the twist angle and distortions of the sugarphosphate backbone (MIROSHNYCHENKO and SHESTOPALOVA 2010; AIROLDI et al. 2014).

Major toxicities associated with doxorubicin are bone marrow suppression, gastrointestinal upset including nausea, vomiting and diarrhoea, and myocardial toxicity, which is cumulative and dose limiting (MAULDIN et al. 1992; CHUN et al. 2007). The potential cardiotoxicity of doxorubicin has been reported in humans (CHLEBOWSKI 1979; SALTIEL and MCGUIRE 1983) and dogs and cats (VAN VLEET et al. 1980; DITCHEY et al. 1984; OGILVIE et al. 1989; MAULDIN et al. 1992; O'KEEFE et al. 1993). The cardiotoxic effects may occur at a cumulative dose beginning at 150-240 mg/m² (VAN VLEET et al. 1980; MAULDIN et al. 1992; KITTLESON and KIENLE 1998b; SISSON et al. 1999). They display as a potentially irreversible cardiomyopathy manifesting as arrhythmias, myocardial failure or both (CHLEBOWSKI 1979; KITTLESON and KIENLE 1998b). The exact mechanism of doxorubicin-induced cardiac toxicity has not been completely elucidated, free radical formation and lipid membrane peroxidation may be involved (SISSON et al. 1999).

While doxorubicin's mechanism of action remains unclear, it is postulated to induce cellular apoptosis via drug-DNA intercalation, as evidenced in affected cells by double-strand breaks (DSBs) in DNA (LI et al. 2008), fragmented nuclei with condensed chromatin, and the formation of apoptotic bodies. Previous studies suggested that doxorubicin's intercalation between DNA base pairs stabilizes the topoisomerase II cleavage complex. Other mechanisms such as lipid peroxidation or DNA damage via activating ataxia-telangiectasia mutated (ATM)-dependent

phosphorylation are also reported to induce apoptosis (TEWEY et al. 1984; HRELIA et al. 2002; HURLEY 2002; KURZ et al. 2004).

Doxorubicin is a protein bound molecule. It is distributed to all parts of the body, except for the central nervous system due to the fact that it cannot pass the blood-brain barrier (SARDI et al. 2014).

After intravenous dosing, doxorubicin blood levels fall dramatically as the drug distributes into tissues, followed by a slow elimination phase due to renal and biliary clearance and metabolism (GUSTAFSON et al. 2002). Doxorubicin metabolism occurs via reduction of a side chain carbonyl group by aldoketo reductases (AHMED et al. 1981), yielding doxorubicinol, and by reductive cleavage of the sugar moiety to form the 7-hydroxy aglycone (PAN and BACHUR 1980). Doxorubicin partitioning from blood to tissues has been shown to correlate with DNA concentration (TERASAKI et al. 1982), and doxorubicin is also known to bind to anionic lipids, particularly cardiolipin (GOORMAGHTIGH et al. 1980; NICOLAY et al. 1984).

The development of multidrug resistance (MDR) in cancer is responsible for high recurrence rates and failure of cancer chemotherapy (MISRA et al. 2014). Occurrence of MDR can be attributed to several mechanisms such as genetic aberrations or by alteration of drug influx or efflux activity (PALAKURTHI et al. 2012). However, over-expression of the drug efflux transporter P-glycoprotein (P-gp) is a key factor contributing MDR in tumor cells. P-gp belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily that is expressed at various levels in a variety of cell types (KLAPPE et al. 2009; ABBASI et al. 2013; QIU et al. 2014). This P-gp encoded by the MDR-1 gene transports a variety of structurally and functionally diverse chemotherapeutic drugs, thereby reducing the accumulation of therapeutic concentration of drugs in MDR cancer cells, resulting in low cancer chemotherapeutic efficacy (SERES et al. 2011).

Malignant lymphoma is one of the most common neoplastic diseases in dogs, showing a disease cases prevalence of up to 30 out of 100.000 dogs presented (MACEWEN 1990). Most combination protocols for the treatment of malignant lymphoma are so-called CHOP-based and use cyclophosphamide, doxorubicin, vincristine and prednisone (KELLER et al. 1993; GARRETT et al. 2002). In general, combination protocols are preferred to monotherapy protocols. The most effective proven protocol is the COP-protocol (including cyclophosphamide, vincristine and prednisolone). Some have expanded this COP-protocol with other effective substances leading to an

increase in clinical remission (NOLTE and NOLTE 2000). The ACOPA-protocol includes alongside cyclophosphamide, vincristine and prednisolone also doxorubicin and L-asparaginase. After a 22 week treatment regime and a nine week treatment intermission, treatment weeks 10-16 are repeated with a nine week interval up to an overall total time of one and a half year (MYERS et al. 1997). The Madison-Wisconsin-protocol includes alongside cyclophosphamide, vincristine and prednisolone also doxorubicin, L-asparaginase, chlorambucil and methotrexate. After a 25 week treatment regime, a two to three week intermission is scheduled followed by a repeat of week 11-25 treatment up to an overall total time of two years (KELLER et al. 1993). The cyclic combination protocol includes alongside cyclophosphamide, vincristine and prednisolone also doxorubicin, L-asparaginase and methotrexate. After a 14 week treatment regime, week 8,10,12 and 14 are repeated up to end of the first year. (NOLTE and NOLTE 2000). A 12 week cyclic combination protocol is also commonly in use, including alongside cyclophosphamide, vincristine and prednisolone also doxorubicin and L-asparaginase (SIMON et al. 2006). In case of relapse, patients are treated either with a second cycle of the initial protocol or a number of different rescue protocols. All the above mentioned protocols use a similar application route and dosage of doxorubicin. Doxorubicin is administered intravenously at a dose of 30 mg/m² and may not exceed a total dose of 180-200 mg/m².

Cardiac Troponin I (cTnI) is nowadays a commonly used cardiac biomarker for detection of cardiomyopathy and cardiac damage and has been reported to be elevated in several diseases (HAMMER et al. 1991; SCHOBER et al. 2002; OYAMA and SISSON 2004; BAUMWART et al. 2007; HAGMAN et al. 2007; OYAMA et al. 2007; DINIZ et al. 2008; LAVECCHIO et al. 2009; FONFARA et al. 2010; TRAFNY et al. 2010; WESS et al. 2010c). It is a polypeptide found specifically in cardiac muscle tissue and is part of the troponin complex (troponin I, C and T) within the sarcomere in myocardial cells and is important for excitation–contraction coupling (WELLS and SLEEPER 2008).

Cardiac ultrasound or echocardiography is a diagnostic technique using ultrasound to display anatomic and physiologic characteristics of the cardiovascular system (FEIGENBAUM et al. 2005b). Echocardiography, which permits relatively complete noninvasive visualization of left ventricular anatomy, is an attractive method for detecting normality or abnormality of the left ventricle in the clinical setting (DEVEREUX et al. 1984). Several echocardiography techniques exist, including M-

mode, two-dimensional echocardiography, three dimensional echocardiography, contrast echocardiography, Doppler echocardiography (including color Doppler, continuous and pulsed-wave Doppler echocardiography) and Tissue Doppler echocardiography among many others (MOISE and FOX 1999; FEIGENBAUM et al. 2005b). Standard M-mode and two-dimensional echocardiography evaluate cardiac chamber anatomy and motion. M(motion)-mode echocardiography was the first widely used form of echocardiography (PICARD 1994). One of the advantages of the M-mode is the sharp axial resolution and high sampling frequency. This allows small and rapidly moving structures to be recognized and to be accurately related to time and ECG. Disadvantages of the M-mode are associated with the fact that this is a single one dimensional beam which focuses on a very small portion of the heart, requiring two-dimensional image for orientation (MOISE and FOX 1999). The two-dimensional echocardiography utilizes an ultrasonic beam moving in a sector displaying more anatomical and functional characteristics than the M-mode (MOISE and FOX 1999). In modern scanners, any of the additional domains of imaging such as M-mode and Doppler can be simultaneously performed and superimposed on the two-dimensional image or otherwise simultaneously displayed (FEIGENBAUM et al. 2005b). Tissue Doppler echocardiography, also known as Doppler myocardial imaging, has been available in clinical ultrasound machines since the mid 1990's (SUTHERLAND et al. 1999). Tissue Doppler imaging is a novel echocardiographic method that derives measurements of contraction and relaxation velocities directly from the myocardium (UEMATSU et al. 1995). Analysis of regional ventricular function is of particular importance because abnormalities may be detected before any change is apparent in measures of global function (ERBEL et al. 1996). Tissue Doppler Imaging (TDI) can be displayed as a color display, saturating the typical anatomic structural information. Virtually any area of the myocardium can be evaluated in this manner (FEIGENBAUM et al. 2005a). TDI determines the direction and velocity of wall motion. Several additional parameters of systolic function can be derived from this velocity determination (FEIGENBAUM et al. 2005a).

One of the most commonly used echocardiographic methods to assess cardiac function is the M-mode based Left Ventricular Fractional Shortening (LVFS) measurement (reference value: 25-45%) (BOON 1998; KITTLESON and KIENLE 1998a; MOISE and FOX 1999; CORNELL et al. 2004). LVFS is easy to measure and a coefficient of variation range of 8.7-14.9 is reported in the literature (CHETBOUL et

al. 2005c). However, the interpretation of LVFS offers only low specificity with regard to LV systolic performance due to its strong dependence on pre- and afterload (KITTLESON and KIENLE 1998a). By using the M-mode echocardiography, the motion of the left ventricular free wall (LVPW) and the interventricular septum (IVS) in relation to one another and to time can be depicted. It can be used to measure left ventricular wall thickness and internal diameters during the diastolic (EDD) and systolic (ESD) phases of the cardiac cycle. The LVFS is the difference between the enddiastolic and endsystolic dimensions divided by enddiastolic dimension. By multiplying it with 100, the FS in percentage can be calculated: $((EDD-ESD)/EDD) * 100$ (BOON 1998; KITTLESON and KIENLE 1998a).

Tissue Doppler Imaging (TDI) and Speckle Tracing are widely used techniques in the evaluation and assessment of myocardial function such as velocity movement, deformation and deformation rate. TDI includes several techniques such as Tissue Velocity Imaging (TVI), Strain and Strain rate measurements (CHETBOUL et al. 2004a; CHETBOUL et al. 2004b; CHETBOUL et al. 2004c; CHETBOUL et al. 2005a; CHETBOUL et al. 2005b; CHETBOUL et al. 2006a; CHETBOUL et al. 2006b; CHETBOUL et al. 2006c; CHETBOUL et al. 2007a; CHETBOUL et al. 2007b; MARGIOCCO et al. 2009; WESS et al. 2010a; SIMAK et al. 2011; WESS et al. 2011). These techniques are used to assess myocardial systolic and diastolic myocardial function in dogs and cats (CHETBOUL et al. 2004a; CHETBOUL et al. 2004b; CHETBOUL et al. 2004c; CHETBOUL et al. 2005a; CHETBOUL et al. 2005b; CHETBOUL et al. 2006a; CHETBOUL et al. 2006b; CHETBOUL et al. 2006c; CHETBOUL et al. 2007a; CHETBOUL et al. 2007b; MARGIOCCO et al. 2009; WESS et al. 2010a; SIMAK et al. 2011; WESS et al. 2011).

The aim of the present study was to examine whether evidence of cardiac toxicity due to doxorubicin usage as a part of the 12 week combination protocol can be detected. The methods of detection were the conventional echocardiographic method of M-mode, retrospectively evaluating patients undergoing chemotherapy treatment with the 12 week combination protocol, and a relatively new method of echocardiography including Tissue Velocity Imaging as well as Ultrasensitive Troponin I.

II. Publications

1. G.Tater, N.Eberle, S. Hungerbueler, AJoetzke, I. Nolte. D. Simon (2011):

Assesment of Fractional Shortening (FS) parameter in 37 dogs with malignant lymphoma undergoing chemotherapy treatment with doxorubicin [abstract].

In: 19. Jahrestagung der FG "Innere Medizin und klinische Labordiagnostik" der DVG (InnLab); 2011 Feb 4-5; Leipzig, Tierärztliche Praxis Kleintiere 2011 39 1: A1-A24

2. TATER, G., EBERLE, N., HUNGERBUEHLER, S., JOETZKE, A., NOLTE, I., WESS, G. and BETZ, D.(2011):

Assessment of Cardiac Troponin I (cTnI) and Tissue Velocity Imaging (TVI) in 14 dogs with malignant lymphoma undergoing chemotherapy treatment with doxorubicin – First Results [abstract].

In: 21st ECVIM-CA Congress – Oral Research Communication; 2011 Sep 8-10; Seville, J Vet Inter Med 2011;25:1470-1509

3. TATER, G., EBERLE, N., HUNGERBUEHLER, S., JOETZKE, A., NOLTE, I., WESS, G. and BETZ, D. (2012):

Ventricular fractional shortening in 108 dogs with malignant lymphoma undergoing chemotherapy with a cyclic combination protocol including doxorubicin.

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The independent contribution to this publication included echocardiography performance, retrospective research of cases, evaluation of results, literature research and writing of the manuscript as the main author.

4. TATER, G., EBERLE, N., HUNGERBUEHLER, S., JOETZKE, A., NOLTE, I., WESS, G. and BETZ, D.(2015):

Assessment of Cardiac Troponin I (cTnI) and Tissue Velocity Imaging (TVI) in 14 dogs with malignant lymphoma undergoing chemotherapy treatment with doxorubicin

Vet Comp Oncol. 10.1111/vco.12135

DOI: 10.1111/vco.12135

The independent contribution to this publication included prospective assignment of patients, echocardiography and ECG performance, sample preservation, archiving and coordination with evaluating laboratory, evaluation of results, literature research and writing of the manuscript as the main author.

III. General discussion

The aim of this work was to evaluate whether changes in the LVFS, cTnI concentration and/or TVI measurements depicting myocardial segments velocity can be detected in dogs with malignant lymphoma undergoing a chemotherapy combination protocol including doxorubicin. In the past, most of the echocardiographic cardiotoxic effects of doxorubicin administration in the veterinary literature were described by LVFS changes. Advancement in diagnostic means over the years and new chemotherapy protocols may shed a new light on doxorubicin cardiotoxicity.

In this work, no significant changes of median LVFS, median cTnI and mean TVI could be demonstrated during a cyclic chemotherapy combination protocol including doxorubicin. None of the included patients, that were divided into four groups according to the number of doxorubicin treatment received, showed a significant change in median LVFS or a decreased median LVFS below the cut-off value of 25% during treatment. Median cTnI value ranged between 0-0.098 ng/ml and did not exceed the cut-off value of 0.2 ng/ml at any time point. This cut off value has been reported for other cTnI measurement kits (OYAMA and SISSON 2004). All TVI measurements remained within the standard deviation reference range.

Several reports of cardiotoxicity associated with doxorubicin administration in the human and veterinary literature exist (CHLEBOWSKI 1979; VAN VLEET et al. 1980; SALTIEL and MCGUIRE 1983; DITCHEY et al. 1984; OGILVIE et al. 1989; MAULDIN et al. 1992; PAGE and KEENE 1992; O'KEEFE et al. 1993; THOMAS et al. 1993; OGILVIE et al. 1996; SELTING et al. 2004; BELHAM et al. 2007). Pericarditis/myocarditis, left ventricular dysfunction and arrhythmias (BELHAM et al. 2007; CHETBOUL et al. 2007a) have been described as well as cardiomyopathy leading to congestive heart failure and considered to be dependent on the doxorubicin cumulative dose (CHLEBOWSKI 1979).

The exact onset of cardiotoxicity is unknown, may occur during or after the course of chemotherapy, effects occurring within three months of the completion of chemotherapy may be considered early (BELHAM et al. 2007).

Mauldin et al. (MAULDIN et al. 1992) reported clinical and pathological cardiac abnormalities in 37 of 135 dogs treated with doxorubicin. These abnormalities included arrhythmias, congestive heart failure, myocardial degeneration, vacuolation, fibrosis and coronary arteriosclerosis. Severe interventricular septum myocytes degeneration, sarcoplasmic reticulum distension and vacuoles are also reported (VAN VLEET et al. 1980; PAGE and KEENE 1992; KITTLESON and KIENLE 1998b).

Sorenmo et al. (SORENMO et al. 2004) examined 20 dogs with hemangiosarcoma undergoing five to seven doxorubicin treatments at a dose of 30 mg/m². Eleven dogs underwent histologic grading of cardiac damage. In five of these eleven dogs, moderate to severe fibrosis and mild to moderate vascular changes were found. No clinical signs of dilated cardiomyopathy or congestive heart failure were documented in these dogs. LVFS, cTnl and TVI changes were not evaluated in this study.

Some authors recommend avoiding doxorubicin treatment if the LVFS is below 20%, and echocardiography controls are recommended starting at a cumulative doxorubicin dose of 90 mg/m². Furthermore, it has been recommended to consider discontinuation of doxorubicin administration if the LVFS measurement falls below 25% (unless no other reasonable alternative treatment is available) (SISSON et al. 1999). One patient from the present study (number 98), an extremely calm Golden Retriever, demonstrated an LVFS of 18% after the second doxorubicin administration. The end-diastolic and endsystolic diameters (EDD and ESD, respectively) were within normal reference values (CORNELL et al. 2004) (EDD: 3.36 cm, ESD: 2.75 cm) and the dog did not show signs of systolic dysfunction, therefore treatment was continued. Subsequently, the same patient demonstrated a normal FS of 28% and 25% during follow-up examinations. One explanation for these findings may be the reported variability of the LVFS measurement between 8.7 to 14.9% (CHETBOUL et al. 2005c). Belham et al. (BELHAM et al. 2007) examined 67 human patients receiving doxorubicin chemotherapy. In this study, six patients had pre-existing cardiac disease. The remaining 61 patients received an average dose of 293 ± 103 mg/m² doxorubicin at the time of completing the study. Ejection fraction (EF) based on the modified Simpson's rule, Tei index, tissue Doppler imaging parameters and traditional systolic and diastolic parameters (including LVFS) were evaluated prior to and following chemotherapy. The best parameter to predict development of functional cardiotoxicity

was the ejection fraction. However, slight changes in the LVFS were also noted in this study.

The measurement of LVFS is one of the most commonly used and well known measurements of cardiac assessment and is relatively rapid and easy to perform (BOON 1998; KITTLESON and KIENLE 1998a; MOISE and FOX 1999; CORNELL et al. 2004). Unfortunately, this measurement depends on pre- and afterload and is not a very specific index of myocardial contractility (KITTLESON and KIENLE 1998a).

Several sensitive laboratory tests, such as the biomarker cTnI exist to evaluate myocardial damage which is increased in cases of myocardial ischemia, congestive heart failure, and myocarditis, but also in some extracardiac conditions such as gastric dilation volvulus and snake bites (OYAMA and SISSON 2004; WELLS and SLEEPER 2008). cTnI is also elevated in cases of specific cardiac diseases such as the Doberman cardiomyopathy (WESS et al. 2010c), bradyarrhythmias (TRAFNY et al. 2010) or in boxers with arrhythmogenic right ventricular cardiomyopathy (BAUMWART et al. 2007) and was reported to be increased in dogs undergoing doxorubicin therapy and experimental intracoronary administration of doxorubicin (SELTING et al. 2004). Selting et al. (SELTING et al. 2004) reported a relative increase of cTnI concentration in lymphoma patients in relation to osteosarcoma patients post chemotherapy with doxorubicin. In this retrospective study 31 dogs were included and 13 dogs were diagnosed with lymphoma. cTnI measurements were performed in six week intervals and no evaluation and/or control of kidney enzyme profile was reported. Ten dogs were diagnosed with evidence of cardiac disease, while only four of these ten dogs had an echocardiographic pre-screening, and it is unclear how many, if any, were lymphoma patients. In this study (SELTING et al. 2004) cTnI could not predict cardiac outcome, but an increase in mean cTnI concentration was reported among groups in comparison to baseline. Nevertheless, mean cTnI concentration during protocols for all dogs was < 0.03 ng/ml.

TDI is commonly used for evaluation of cardiac function, systolic and diastolic functions, including tissue velocity imaging, strain, strain rate and speckle tracking (CHETBOUL et al. 2007b; KILLICH et al. 2011; SIMAK et al. 2011).

A slight reduction in early diastolic phase velocity in relation to late diastolic phase velocity was noted after the first doxorubicin administration. A negative influence of

age on the early diastolic phase detected by mitral flow velocities using a conventional Doppler examination as well as TVI and Strain rate have been reported (CHETBOUL et al. 2005b; SIMAK et al. 2011). Median age of the prospectively included population of dogs is 8.9 years, showing that this population is of relative advanced of age. The inter- and intraobserver coefficient of variation for the TVI method was proven to be good (< 9 %) (SIMAK et al. 2011), it is plausible that this measurement falls within this 9 % explaining the slight reduction documented in the relation between the early and late diastolic phases.

The lack of significant changes in LVFS, TVI and Troponin I documented here may be due to several reasons. The elected measurements may not be sensitive enough to detect doxorubicin-induced effects in this chemotherapy protocol. Alternatively, the cumulative dose reached in this study (150 mg/m² or more) may not be high enough to cause significant changes in the LVFS, cTnI or TVI measurements. Thirdly, the method of doxorubicin administration may play an important role. Some reports of toxicity were based on older protocols administrating doxorubicin as a bolus rather than a continuous rate infusion as used in this protocol, or on experimental protocols with intracoronary administration (CHLEBOWSKI 1979; DITCHEY et al. 1984; SHAH et al. 1997; TOYODA et al. 1998).

In this work, a maximum of four consecutive administrations of 30 mg/m² doxorubicin (a cumulative dose of 120 mg/m²) were evaluated. Two more doxorubicin doses were administered to dogs experiencing a recurrence of the disease with an ultimate cumulative dose of up to 180 mg/m². This fifth or even sixth doxorubicin administration may occur at an unscheduled time point according to the patient's general state and state of remission. As a relative long time may elapse between the fourth and the following renewed doxorubicin, these treatments were not evaluated in this study.

There are however several limitations to this work. One of them is the retrospective nature of the first study. Here examination was performed by different investigators, and no intra- and interobserver analysis or reproducibility evaluation was possible. Prospective studies evaluating intra- and interobserver variations as well as measurement reproducibility are warranted to verify the observations of this study. Another limitation of this work is that the potential arrhythmogenic effect of doxorubicin

was not evaluated. A short term ECG was performed for every patient in the prospective study were found normal, but no long-term evaluation including 24-hour “Holter” ECG was performed. Another limitation that must be considered is that TVI cannot differentiate between passive and active movement of the different segments and can be influenced by tethering effects. In contrast, Strain and Strain rate measurements of myocardial segments have been reported in the human literature as sensitive indicators for myocardial damage and disease (SUTHERLAND et al. 2004; DANDEL and HETZER 2009) and are not influenced by tethering effects. In this work all patients were treated with the cyclic combination protocol consisting of four different drugs. Although only doxorubicin is reported to have a cardiotoxic effect, it is not possible to rule out that another drug or a combination of drugs could be responsible for an effect on cardiac function. Other limitations of the prospective study presented in this work originate from the small number of patients included. No other parameter but LVFS was evaluated in the retrospective study presented in this work. A complete evaluation of cardiac function however includes many parameters that are less pre- and afterload-dependent parameters evaluating systolic and diastolic functions (such as enddiastolic and endsystolic diameters, normalised diameter indexes, tissue velocity imaging, etc.). Due to the retrospective nature of this study, not all of these parameters are available.

This current work presented here is the first in the literature reporting serial LVFS measurements and prospective ultrasensitive cTnl and TVI measurements in dogs with malignant lymphoma undergoing chemotherapy treatment with a cyclic combination protocol including doxorubicin.

Since the evaluation of cardiac function, in the veterinary practice, is commonly performed by LVFS measurement and nowadays increasingly also by cTnl measurement and TVI technique these methods were chosen to be evaluated in the present work.

Future larger prospective studies should evaluate the efficacy of other less commonly used testing methods such as Strain, Strain rate or speckle tracking method (CHETBOUL et al. 2007a; KILLICH et al. 2011; SIMAK et al. 2011), as well as late effects of doxorubicin in the analysis of chemotherapy induced cardiotoxicity. The use of the Simpson’s method of disc is already applied in today’s veterinary medicine and could also be used in the investigation of doxorubicin effects on the cardiac muscles in

animals (WESS et al. 2010b). Variability of the latter method is reported to be smaller than LVFS (WESS et al. 2010b).

In contrast to today's evaluation, only LVFS was considered to be necessary for the evaluation of cardiotoxicity and cardiac contractility evaluation in the past.

The findings of this current work show that the 12 week chemotherapy protocol is a safe protocol to be used from a cardiac point of view, since all methods failed to show an evidence of cardiac damage caused by the protocol. Since no pathological examinations exist, cellular level damage cannot be ruled out, but if present its clinical importance is not evident in these studies.

IV. Summary

Evaluation of the cyclic combination chemotherapy protocol including doxorubicin effects on cardiac parameters

Guy Tater

Doxorubicin is a commonly used chemotherapy agent for the treatment of canine malignant lymphoma. It is reported to induce cardiotoxic effects when administered in high doses. The aim of this work was to examine whether any cardiac effects can be documented following doxorubicin administration as part of a cyclic chemotherapy protocol. Dogs were recruited for this work after being diagnosed with canine malignant lymphoma and undergoing a cyclic combination chemotherapy protocol including L-asparaginase, vincristine, cyclophosphamide, prednisolone and doxorubicin.

The first part of this work retrospectively analysed the echocardiographic records of 108 patients. LVFS as the most commonly reported and used method to detect doxorubicin cardiotoxicity, was examined.

Records of dogs presented between the years 2001 to 2010 were reviewed and 446 LVFS measurements from 108 dogs were included. No significant change of the LVFS was documented along the different stages of the chemotherapy protocol. The lower reference value used for LVFS of 25% was not exceeded.

The second part of this work included a prospective study. The aim of this study was to use more advanced and sensitive methods to detect possible cardiac changes and/or damage during the cyclic combination protocol. Fourteen dogs were included. Blood work was performed prior to inclusion to exclude kidney damage. All dogs underwent an echocardiography examination and ultrasensitive Troponin I sampling prior and post doxorubicin administrations. The echocardiographic examination included standard as well as tissue velocity imaging measurements.

Systolic and diastolic velocity images were examined on interventricular septum and free wall. A total of 182 TVI and 1017 ultrasensitive Troponin I measurements were performed and evaluated. During the twelve week cyclic combination protocol, Tissue Doppler indices and ultrasensitive Troponin I measurements did not change

significantly.

These findings conclude that the cyclic combination protocol seems to be safe, and no evidence of myocardial damage was detectable using echocardiographic or ultrasensitive Troponin I measurements. Further larger prospective studies should evaluate suspected doxorubicin induced myocardial damage, using sensitive new methods such as strain, strain rate and speckle tracking. Myocardial damage at the cellular level could not be excluded but if present it did not appear to have a clinical impact on the population of dogs included in this work.

V. Zusammenfassung

Evaluierung des Effekts eines Doxorubicin-beinhaltenes Zyklischen Kombinationschemotherapie-Protokolls auf kardiale Parameter.

Guy Tater

Doxorubicin ist zur Behandlung des malignen Lymphoms des Hundes ein häufig angewendetes Chemotherapeutikum. In hohen Dosen verabreicht kann eine kardiotoxische Wirkung beobachtet werden. Ziel dieser Arbeit war es zu untersuchen ob, bei Anwendung eines zyklischen Chemotherapie-Protokolls zur Behandlung des canines Lymphoms ein Effekt auf das Herz nach Anwendung dieses Medikaments festgestellt werden kann. Hunde, bei denen die Diagnose canines malignes Lymphom gestellt wurde und die mit einem zyklischen Chemotherapie-Protokoll mit L-Asparaginase, Vincristin, Cyclophosphamid, Prednisolon und Doxorubicin behandelt wurden, wurden in die Studie eingeschlossen.

Im ersten Teil dieser Arbeit erfolgte die retrospektive Auswertung und Analyse von echokardiographischen Aufnahmen, wobei eine mögliche doxorubicin-induzierte Kardiotoxizität anhand der LVFS untersucht wurde.

Die Aufnahmen von zwischen 2001 und 2010 vorstellig gewordenen Hunden wurden untersucht und insgesamt 446 LVFS Messungen von 108 Hunden in die Studie eingeschlossen. Es konnte kein signifikanter Unterschied der LVFS in den verschiedenen Stadien des chemotherapeutischen Protokolls festgestellt werden. Der gebräuchliche Referenzwert der LVFS von mindestens 25 % wurde nicht unterschritten.

Der zweite Teil dieser Arbeit umfasste eine prospektive Studie. Ziel dieser Studie war es, sensitivere und ausgereifere Methoden, die mögliche Veränderungen bzw. Schäden am Herzen durch das zyklische Kombinationsprotokoll feststellen sollten, zu evaluieren. Vierzehn Hunde wurden eingeschlossen, bei denen zuvor erhöhte Nierenwerte anhand eines Blutbildes ausgeschlossen worden waren. Des Weiteren wurde von allen Studienteilnehmern ein Herzultraschall angefertigt und ultrasensitives Troponin I vor und nach Doxorubicin-Gabe gemessen.

Die Ultraschalluntersuchung beinhaltete Standard- und Gewebedoppler-Messungen. Systolische und diastolische Gewebegeschwindigkeiten des interventrikulären Septums und der freien Wand wurden untersucht. Insgesamt wurden 182 Gewebegeschwindigkeits- und 1017 ultrasensitive Troponin I Messungen durchgeführt und bewertet.

Während des zwölfwöchigen zyklischen Kombinationsprotokolls wiesen weder die Gewebedoppler-Messungen noch die Messungen des ultrasensitiven Troponin I signifikante Unterschiede auf.

Ob Doxorubicin einen myokardialen Schaden verursacht sollte anhand weiterführender prospektiver Studien beurteilt werden, in denen andere sensitive Messmethoden wie die Strain, Strain Rate und das Speckle Tracking zum Einsatz kommen. Auch wenn es innerhalb der Studienpopulation keinen Hinweis gab, lässt sich ein Schaden des Myokards auf zellulärer Ebene durch die eigene Studie nicht mit Sicherheit ausschließen.

VI. Literature

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Recent attempts at RNAi-mediated P-glycoprotein downregulation for reversal of multidrug resistance in cancer.

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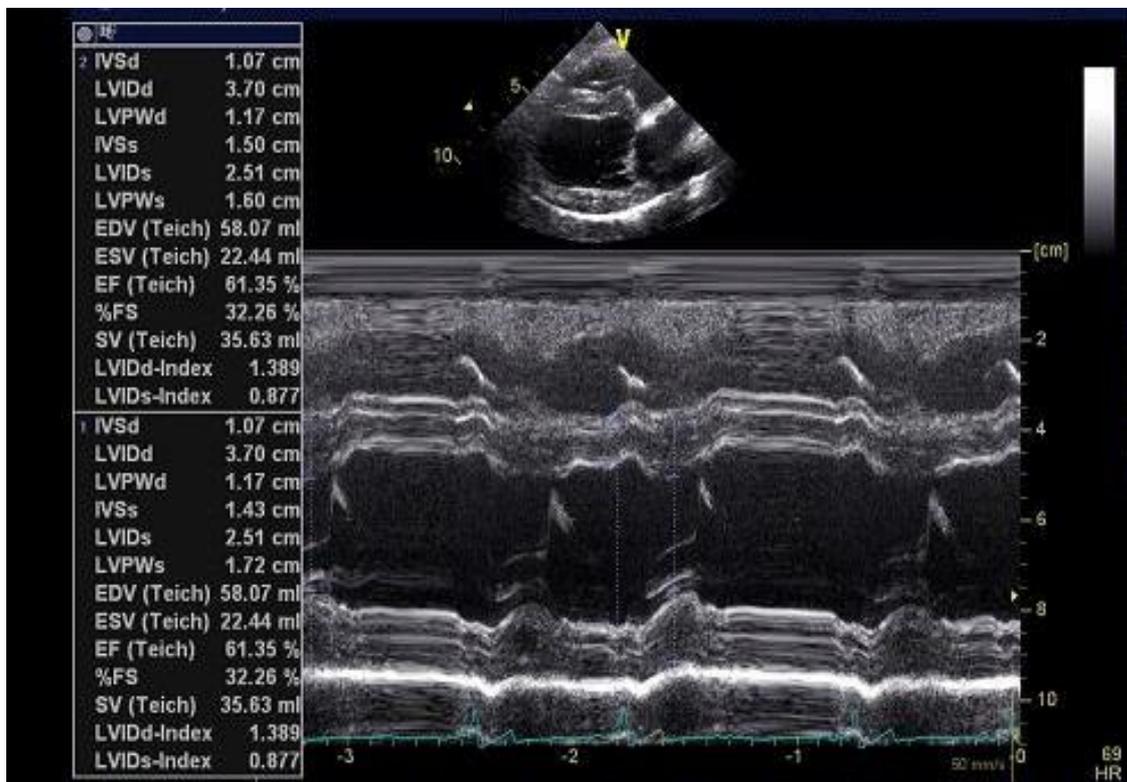
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VII. Tables and figures for Chapter II.1

Abb. 1 Right parasternal long axis view depicting an M-Mode. The cursor is placed along the long and short axis view at end diastole and end systole. IVSd = intraventricular septum diameter in diastole, IVSs = intraventricular septum diameter in systole, LVIDd = left ventricular inner diameter in the diastole, LVIDs = left ventricular inner diameter in the systole, LVPWd = left ventricular posterior wall diameter in diastole, LVPWs = left ventricular posterior wall diameter in systole, FS = left ventricular fractional shortening.



Tab. 1 12-week chemotherapy protocol used for the treatment of canine lymphoma as described by Simon et al.

Drug	Dose	Week														
		1	2	3	4	5	6	7	8	9	10	11	12	13		
L-asparaginase ^a	400 IU/kg SC	•														
Vincristine	0.7 mg/m ² IV	•			•			•			•					
Cyclophosphamide	200 mg/m ² IV		•			•			•				•			
Doxorubicin ^b	30 mg/m ² IV*			•			•			•				•		
Prednisolone	50 mg/m ² PO q24h for 3 days	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Echocardiography		•			•			•			•					•

IV, intravenous administration; IV*, intravenous infusion over 30 min; PO, oral administration

^aPretreatment with prednisolon

^bAdministration of prednisolone on days 1-3

Tab. 2 Table showing left ventricular fractional shortening (LVFS) changes of the four groups during the cyclic combination chemotherapy protocol including doxorubicin.

Groups	Measurement	LVFS (min-max)
Group 1 (one DT) (n = 7)	median baseline LVFS (%)	33 (26-40)
	median LVFS post 1 DT (%)	30 (27-37)
Group 2 (two DT) (n = 17)	median baseline LVFS (%)	33 (20-49)
	median LVFS post 1 DT (%)	34 (25-43)
	median LVFS post 2 DT (%)	31 (25-43)
Group 3 (three DT) (n = 39)	median baseline LVFS (%)	35 (24-50)
	median LVFS post 1 DT (%)	34 (25-50)
	median LVFS post 2 DT (%)	34 (25-50)
	median LVFS post 3 DT (%)	32 (22-43)
Group 4 (four DT) (n = 45)	median baseline LVFS (%)	32 (22-49)
	median LVFS post 1 DT (%)	32 (24-54)
	median LVFS post 2 DT (%)	35 (18-54)
	median LVFS post 3 DT (%)	32 (25-54)
	median LVFS post 4 DT (%)	32 (20-48)
DT = doxorubicin treatment(s)		

Tab 3 Results from repeated measures. ANOVA test assessing significance changes in left ventricular fractional shortening (LVFS) during a cyclic combination protocol including doxorubicin treatments. $P < 0.05$ is considered significant.

Group	Repeated measures ANOVA	P-value
Group 1 (one DT) (n = 7)	baseline – 1st DT	0.33
Group 2 (two DT) (n = 17)	baseline – 1st DT	1.0
	baseline – 2nd DT	1.0
	1st – 2nd DT	0.078
Group 3 (three DT) (n = 39)	baseline – 1st DT	1.0
	baseline – 2nd DT	1.0
	baseline – 3rd DT	0.053
	1st – 2nd DT	1.0
	1st – 3rd DT	0.078
	2nd – 3rd DT	0.615
Group 4 (four DT) (n = 45)	baseline – 1st DT	1.0
	baseline – 2nd DT	1.0
	baseline – 3rd DT	1.0
	baseline – 4th DT	1.0
	1st – 2nd DT	1.0
	1st – 3rd DT	1.0
	1st – 4th DT	1.0
	2nd – 3rd DT	0.1
	2nd – 4th DT	0.877
	3rd – 4th DT	1.0
DT = doxorubicin treatment(s)		

VII. Tables and figures for Chapter II.2

Table 1: 12- week chemotherapy protocol used for the treatment of canine lymphoma as described by Simon et al 2006

Drug	Dose	Week													
		1	2	3	4	5	6	7	8	9	10	11	12	13	
L-asparaginase ^a	400 IU/kg SC	•													
Vincristine	0.7 mg/m ² IV	•			•			•			•				
Cyclophosphamide	200 mg/m ² IV		•			•			•			•			
Doxorubicin ^b	30 mg/m ² IV*			•			•			•			•		
Prednisolone	50 mg/m ² PO q24h for 3 days	•	•	•	•	•	•	•	•	•	•	•	•	•	
Echocardiography		•			•			•			•			•	

IV, intravenous administration; IV*, intravenous infusion over 30 min; PO, oral administration

^aPretreatment with prednisolon

^bAdministration of prednisolone on days 1-3

Table 2: Patient characteristics of 14 patient dogs with malignant lymphoma undergoing chemotherapy treatment with cyclic combination protocol.

n = 14		range
Median age (years)	8.9	4.5-13.6
Median weight(kg)	29.9	4.4-40
Male/female	7/7	
Overall number of cTnl measurements	121	
Overall number of TVI measurements	1017	
B lymphoma	11/14 (78.6%)	
T lymphoma	2/14 (14.3%)	
Unclassified lymphoma	1/14 (7.1%)	
Anatomical classification	12 multicentric, 1 intestinal, 1 epitheliotropic	
Clinical stages	3a n = 1 (7.1%) 3b n = 1 (7.1%) 4a n = 4 (28.6%) 4b n = 2 (14.3%) 5a n = 2 (14.3%) 5b n = 4 (28.6%)	

Table 3: Median cTnl results according to chemotherapy/visit week. Colored row/boxed represents TVI measurement at the given time. Significant difference $P < 0.05$; $P = 0.421$.

Chemo Week	1	2	3	4	5	6	7	8	9	10	11	12	13
Median cTnl	0.022	0.00	0.00	0.00	0.041	0.068	0.061	0.096	0.057	0.054	0.073	0.083	0.098

Table 4: Tissue Velocity Imaging results in 14 dogs undergoing chemotherapy treatment including doxorubicin.

	Mean value IVS-S	Mean value IVS-E	Mean value IVS-A	Mean value LVFW-S	Mean value LVFW-E	Mean value LVFW-A
Mean Reference range cm/s	3.53 - 9.39	(-)5.77 - (-)2.63	(-)4.89 - (-)1.75	(-)7.96 - (-)3.93	(-)11.74 - (-)4.68	(-) 7.32 -(-)2.44
Pre doxo (SD)	7.51 (2.6)	-4.63 (1.73)	-4.5 (1.8)	7.88 (2.12)	-8.64 (2.48)	-6.15 (2.47)
Post 1 doxo (SD)	7.85 (2.53)	-4.54 (1.6)	-4.84 (1.34)	6.8 (2.45)	-7.15 (3.73)	-6.1 (3.1)
Post 2 doxo (SD)	6.58 (1.87)	-4.78 (1.03)	-4.48 (1.56)	7.1 (2.81)	-7.57 (3)	-6.65 (2.45)
Post 3 doxo (SD)	6.56 (2.08)	-5.1 (1.06)	-4.25 (1.14)	6.69 (1.75)	-7.14 (2.34)	-5.8 (2.05)
Post 4 doxo (SD)	5.88 (1.5)	-4.32 (0.98)	-3.8 (1.55)	6.01 (2.65)	-7.08 (2.68)	-5.51 (2.28)
P =	0.353	0.562	0.373	0.36	0.574	0.848
Significant P <0.05						

IVS- Interventricular Septum, LVFW – Left Ventricular Free Wall, S – systole, E- Early diastolic wave, A – Late diastolic wave, SD – Standard Deviation, doxo – doxorubicin administration. Reference range - Killich M, Hartmann K et al. Tissue velocity imaging in healthy dogs. *Tierärztliche Praxis Kleintiere*2011; **39**(2): 69-78.

Figure 1: Left apical four chamber view depicting the left ventricle. 1a - a typical TVI curve in left ventricular free wall basal segment showing an S, E and A wave. 1b - denotes left ventricle divided into six different segments – two basal, two middle and two apical segments (yellow markers).

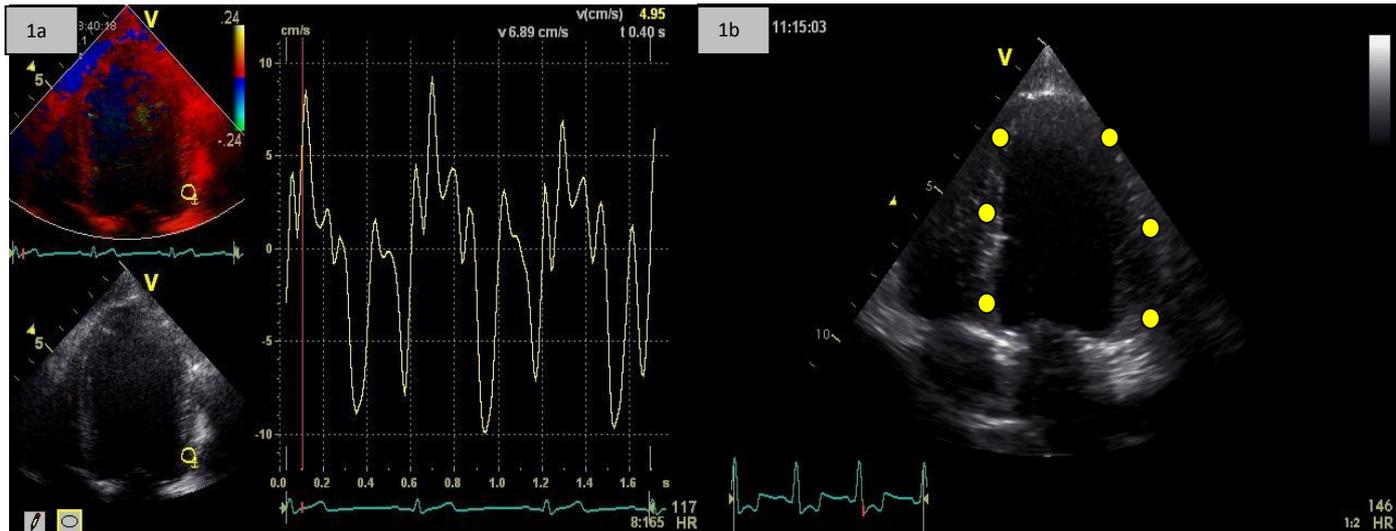


Figure 2: Box plot multiple comparison graph depicting median cTnI measurements of patients (n=14) during the weekly chemotherapy protocol. No statistically significant difference was seen between the groups (P=0.421)

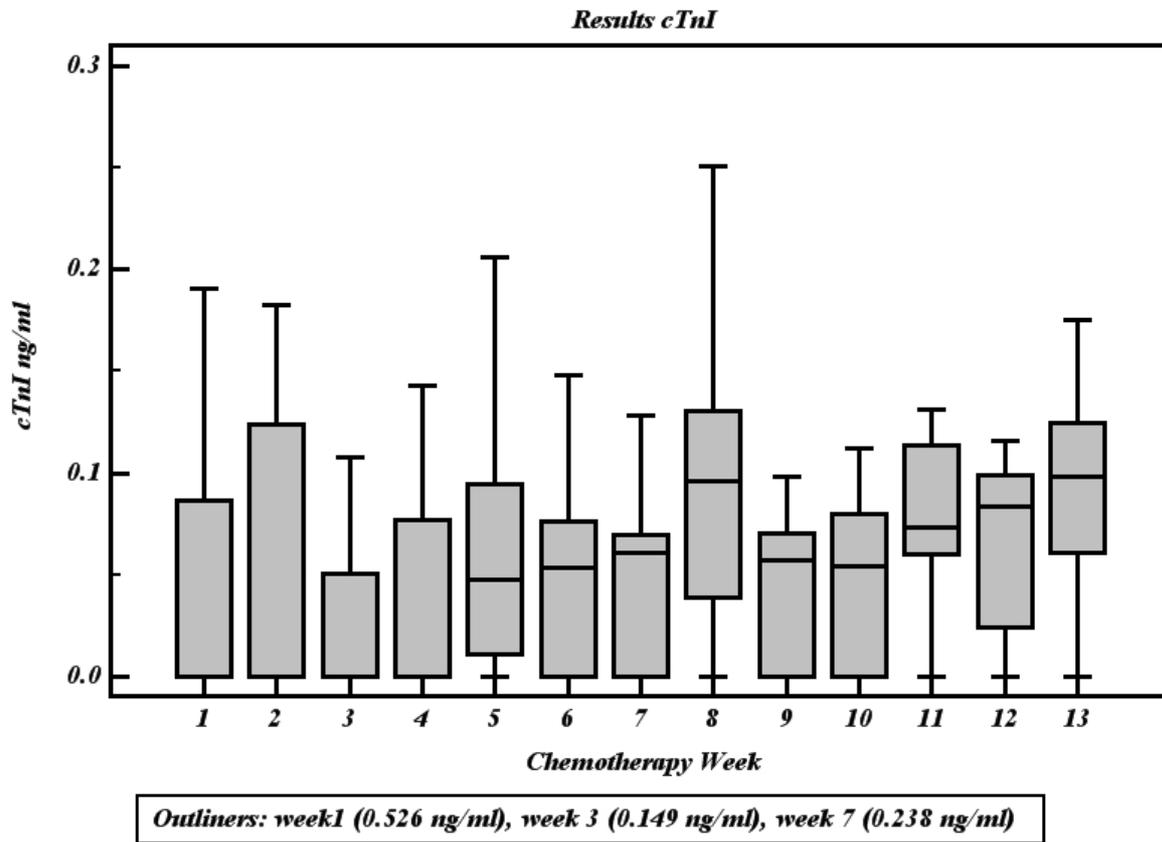


Figure 3: Box plot multiple comparison graph depicting mean interventricular septum TVI measurements in systole and 2 diastolic phases (E wave- chequered, A wave – striped) of included patients (n=14) during the chemotherapy weeks.

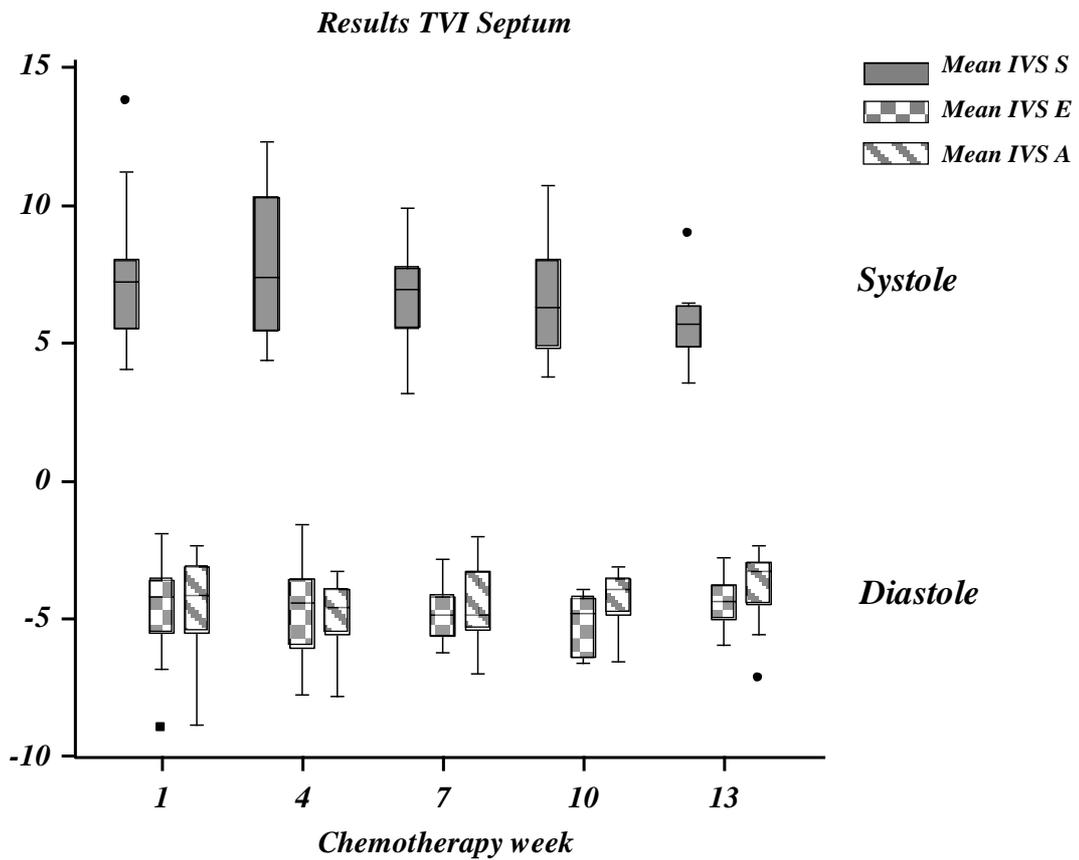
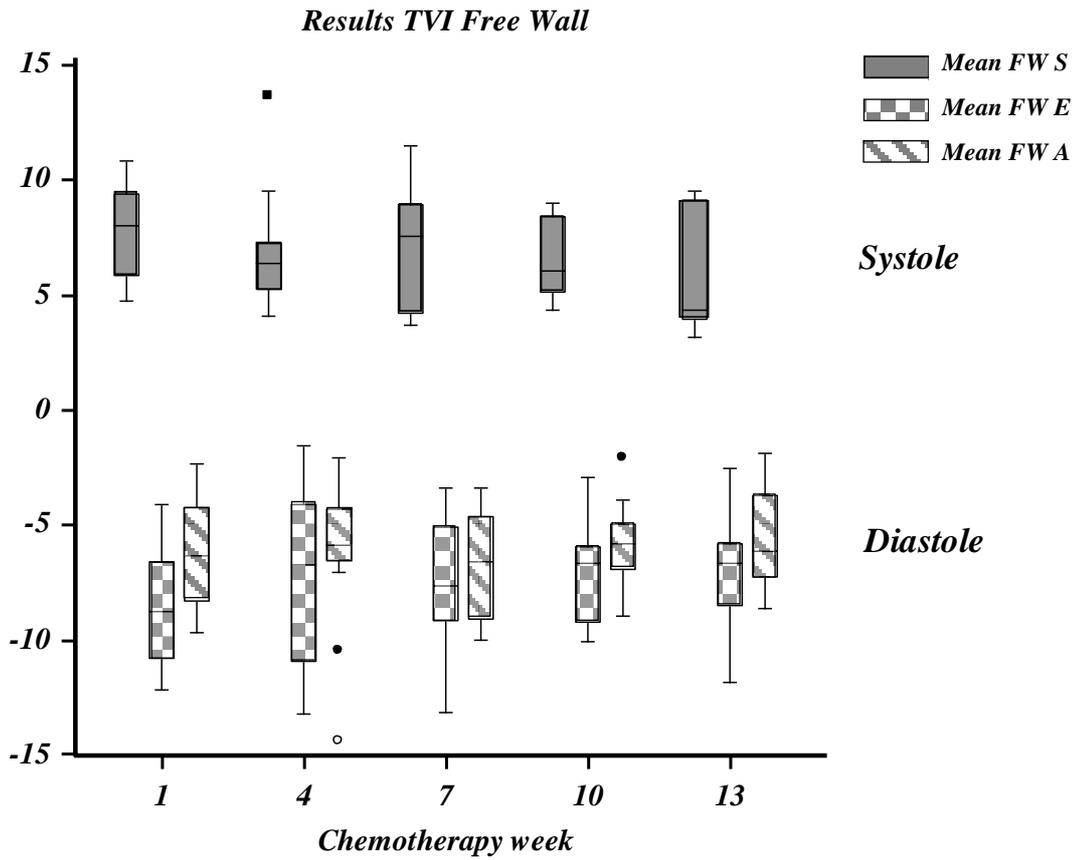


Figure 4: box plot multiple comparison graph depicting mean left ventricular free wall TVI measurements in systole and 2 diastolic phases (E wave- chequered, A wave – striped) of included patients (n=14) during the chemotherapy weeks protocol.



IX. List of abbreviations

cTnI	cardiac Troponin I
ECG	Electrocardiography
EDD	End Diastolic Diameter
ESD	End Systolic Diameter
FS	Left ventricular Fractional Shortening
IVS	Interventricular Septum
IVSd	Intraventricular Septum diameter in diastole
IVSs	Intraventricular Septum diameter in systole
LVIDd	Left Ventricular Inner Diameter in the diastole
LVIDs	Left Ventricular Inner Diameter in the systole
LVFS	Left Ventricular Fractional Shortening
LVPWd	Left Ventricular Posterior Wall diameter in diastole
LVPWs	Left Ventricular Posterior Wall diameter in systole
TDI	Tissue Doppler Imaging
TVI	Tissue Velocity Imaging

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