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Cassandra Linder

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Supervisor:	**Henrik Rönnberg, Swedish University of Agricultural Sciences, Department of Clinical Sciences**
Assistant supervisor:	Sara Saellström, Swedish University of Agricultural Sciences, University Animal Hospital
Assistant supervisor:	Magnus Åbrink, Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health
Examiner:	Odd Höglund, Swedish University of Agricultural Sciences, Department of Clinical Sciences

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Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences, Oncology

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Abstract

Hemangiosarcoma (HSA) is a common splenic malignant neoplasia in dogs, originating from the endothelium. The tumor has an aggressive character, and the clinical symptoms of HSA are mostly due to the secondary effects of the tumor such as tumor growth or rupture. The prognosis is poor for HSA, usually with a survival time of under one year.

It is challenging to distinguish HSA from benign neoplasias in the spleen, such as hematoma, which has a significantly better prognosis. Hence, many dogs are euthanized today due to suspicion of splenic HSA. With better diagnostic methods, several of these dogs could be cured as the tentative diagnosis may not be correct.

Biomarkers have been discussed as a diagnostic method for HSA. As of today, few biomarkers have been evaluated and somewhat successfully implemented as a diagnostic method in the clinic. Serglycin is a proteoglycan that is mainly expressed by immune cells and have shown, in humans, a correlation between increased expression and malignant cancers. Hence, a quantification of serglycin in tumor canine patients could thus indicate tumor malignancy and be used for diagnostic purposes.

In this study, serglycin expression was analyzed in splenic HSA tissue and non-pathological splenic tissue from formalin-fixed and paraffin-embedded material from dogs. Another gene, Eukaryotic elongation factor 2 (EEF2), was also included in the study to be compared with the expression of serglycin. The analysis was performed in real time with quantitative polymerase chain reaction (qPCR), where the expression in the tumor samples were compared with the healthy samples.

Even though the quality of the qPCR results from the samples varied, this thesis still shows that there are trends that are interesting to continue to investigate with other methods and a larger patient material as a basis. Based on the samples showing the correct expression, this study support that splenic HSA tissue expresses significantly higher levels of serglycin than the non-pathological splenic tissue. This was not the case for EEF2, suggesting that serglycin is a more suitable biomarker for HSA than EEF2. However, before serglycin can be used as a biomarker at the clinic, more samples need to be analyzed to map out the normal variation in dogs. Also, as HSA was now compared with healthy spleens, it would be valuable to compare splenic HSA with other benign splenic masses as well. Lastly, a simple and inexpensive way of measuring the serglycin expression needs to be developed.

Keywords: Serglycin, EEF2, Cancer, Hemangiosarcoma, dog, canine, metastatic, biomarker, qPCR

Sammanfattning

Hemangiosarkom (HS) är en vanlig malign neoplasi i mjälten hos hundar som härrör från endotelet. Tumören har en aggressiv karaktär och de kliniska symtomen på HS beror främst på tumörens sekundära effekter såsom tumörtillväxt eller bristning. Prognosen är dålig för HS, vanligtvis med en överlevnadstid på under ett år.

När det gäller massor i mjälten är det utmanande att skilja HS från benigna neoplastiska förändringar, såsom hematom, som har en betydligt bättre prognos. Vid misstanke om HS i mjälten avlivas många hundar idag, då prognosen är dålig samt operationen kostsam. Med bättre diagnostiska metoder skulle flera av dessa hundar kunna botas eftersom den preliminära diagnosen kanske inte är korrekt.

Biomarkörer har diskuterats som en diagnostisk metod för HS. Dock har få biomarkörer utvärderats, samt framgångsrikt implementerats, som diagnostisk metod på kliniknivå idag. Serglycin är en proteoglykan som huvudsakligen uttrycks av immunceller och som hos människor har visat ett samband mellan ökat uttryck och maligna cancerformer. En kvantifiering av serglycin hos tumörpatienter hos hundar skulle således kunna indikera maligna tumörer och användas för diagnostiska ändamål.

I denna studie analyserades serglycin uttrycket i vävnadsprover från mjältar med HS samt från frisk mjältvävnad från formalinfixerat och paraffininbäddat material från hundar. En annan gen, ”Eukaryotic elongation factor 2” (EEF2), inkluderades även i studien för att jämföras med uttrycket av serglycin. Analysen utfördes i realtid med " quantitative polymerase chain reaction” (qPCR) där uttrycket i tumörproverna jämfördes med den friska mjältvävnaden.

Resultaten från denna studie visar att mjältvävnad med HS uttrycker högre nivåer av serglycin jämfört med frisk mjältvävnad, vilket är i linje med resultat från tidigare studier på människor. EEF2 visade inte ett signifikant högre uttryck i mjältvävnaden med HS jämfört med den friska mjältvävnaden. Detta indikerar att serglycin är en mer lämplig biomarkör för HS än EEF2. Dock behöver fler prover analyseras och jämföras med normal vävnad för att få mer kunskap om normal-variationen i serglycin uttrycket hos hundar. Eftersom HSA nu jämfördes med friska mjältar, skulle det också vara värdefullt att jämföra serglycin uttrycket från HS med andra benigna mjältmassor. Slutligen måste även ett enkelt och billigt sätt att mäta uttrycket utvecklas.

Nyckelord: Serglycin, EEF2, Cancer, Hemangiosarkom, hund, hund, metastatisk, biomarkör, qPCR

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Abbreviations

HSA	Hemangiosarcoma
SLU	Swedish University of Agricultural Sciences
SGN	Serglycin
EFF2	Eukaryotic elongation factor 2
HPRT	Hypoxanthine phosphoribosyl transferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RPS5	Ribosomal Protein S5
qPCR	Quantitative polymerase chain reaction

1. Introduction

Hemangiosarcoma (HSA) is a common splenic malignant neoplasia in dogs, originating from the endothelium. The most common primary sites are spleen, heart and liver. The tumor has an aggressive character; it is proliferative and infiltrating, and often metastasizes early in the course of the disease. The clinical symptoms of HSA are mostly due to the secondary effects of the tumor, such as tumor growth or rupture. HSA of the spleen can cause spontaneous bleeding, anemia, arrhythmia and sudden collapse (Zachary 2017; Nelson & Couto 2019). It is clearly overrepresented among German Shepherds and Golden Retrievers aged 8 to 10 years (Spangler & Kass 1997). The prognosis is poor for HSA, usually with a survival time of under one year (Batschinski et al. 2018).

Today, many dogs are euthanized due to suspicion of splenic HSA as the operation is costly and has a poor prognosis. However, the prognosis is good for benign changes such as hematomas. For diagnosing HSA today, the parameters used are mostly clinical features, imaging as well as blood samples (Johnson *et al.* 1989; Thamm *et al.* 2012; Cleveland & Casale 2016; Nelson & Couto 2019). However, it is challenging to confirm a diagnosis on these parameters, and to reach a more definite diagnosis, histopathology is required (Mallinckrodt & Gottfried 2011; Nelson & Couto 2019). The recommended treatment for dogs with splenic masses is total splenectomy. Chemotherapy may be used as an adjuvant post-operative treatment, or as a palliative treatment for some dogs with multiple masses (Nelson & Couto 2019). With better diagnostic methods, and hence a better base for decision for treatment and surgery, several of these dogs could be cured as the tentative diagnosis may not be correct.

Biomarkers have been discussed as a diagnostic method for HSA. However, few biomarkers have been evaluated and somewhat successfully implemented as a diagnostic method in the clinic as of today (Bryan 2016; Rozolen *et al.* 2021). Serglycin is a proteoglycan that is mainly expressed by immune cells (Korpetinou *et al.* 2014). Recent studies, in human cancers, have shown a correlation between increased expression of serglycin from tumor cells and malignant cancer (Korpetinou *et al.* 2013, 2015). Hence, a quantification of serglycin in tumor canine patients could thus indicate tumor malignancy and be used for diagnostic purposes.

The aim of this project is to help to increase the understanding of the possibilities of serglycin to be used as a diagnostic biomarker for cancer in dogs, by investigating

whether serglycin levels in formalin-fixed paraffin-embedded (FFPE) splenic tissues from dogs with HSA, are increased compared with FFPE samples from dogs with non-pathological spleens.

The method used was quantitative polymerase chain reaction (qPCR) on serglycin expression from tissues from dogs with HSA, as well as non-pathological spleens, from the pathologist's archive of paraffin-embedded material. Twenty-six individuals were analyzed - 20 with HSA and 7 with non-pathological spleens. Reference genes were also included in the study.

2. Literature review

2.1. Splenic masses: etiology, prognosis & management

Masses in the spleen can be divided into two categories: neoplastic and non-neoplastic. Examples of non-neoplastic changes in the spleen are splenic abscesses and hyperplasia. The neoplastic changes on the other hand can be divided into benign and malignant neoplasms, of which benign are for example, hemangiomas, leiomyomas, and myelolipomas. Examples of malignant splenic tumors are leiomyosarcoma, fibrosarcoma, lymphosarcoma and metastatic sarcoma or carcinoma (Leyva *et al.* 2018; Nelson & Couto 2019).

Studies have shown, that in dogs with splenomegaly, approximately 50% are non-neoplastic changes, whereas 50% are neoplastic. Of the neoplastic changes, about 10% are benign and 90% malignant. Of the malignancies, HSA is the most common one. Of the non-malignant diseases, nodular hyperplasia and hematoma are the most common diseases (Spangler & Kass 1997; Eberle *et al.* 2012). In cases of ruptured masses resulting in hemoperitoneum, the chance of malignant neoplasms is significantly higher (Cleveland & Casale 2016; Stewart *et al.* 2020).

The prognosis differs strongly between the splenic masses. For non-neoplastic changes, the survival time after 12 months was 64% while this was 7% for dogs with HSA (Spangler & Kass 1997). Both benign masses as well as malign masses can lead to rupture and further to hypovolemia, decreased tissue perfusion and hypovolemic shock. In mild blood loss, this can be partially or completely compensated for by increased hematopoiesis. When the blood loss is about 35-45%, the blood pressure and stroke volume can decrease drastically. This can result in symptoms such as hypotension, weak pulse, tachycardia, hyperventilation, decreased urination and hypothermia. Other clinical more diffuse signs that can be seen are lethargy, anorexia, vomiting, decreased appetite, signs of heart failure, swollen bones, DIC and pale mucous membranes (Nelson & Couto 2019). Organ failure and systemic effects are obtained at a later stage, and then more organ-

specific symptoms can be seen. In worst case, ruptures of the splenic hematoma or HSA of the spleen can lead to sudden death due to severe hypovolemic shock (Eberle *et al.* 2012; Zachary 2017). When it comes to treatment, surgery in the form of total splenectomy is recommended (Nelson & Couto 2019).

2.1.1. Hemangiosarcoma (HSA)

HSA is a common type of neoplasia and is clearly overrepresented among German Shepherds, Labrador Retrievers and Golden Retrievers aged 8 to 10 years (Spangler & Kass 1997; Nelson & Couto 2019). It is a malignant tumor that originates from the endothelium, and it can originate in principle wherever blood vessels are found, for example in the spleen, liver, intestines, heart and lungs. The tumor has an aggressive character; it is proliferative and infiltrating, and often metastasizes early in the course of the disease and can cause bleeding and hemoabdomen. Hematogenously, the most common sites of metastasis include the liver, peritoneum, mesentery and lungs (Nelson & Couto 2019). Spreading to the auricle of the right atrium is also common. HSA is one of the most common causes of splenectomy (Spangler & Kass 1997)

Histologically, neoplastic endothelial cells wrapped around stroma forming poorly defined blood-filled vascular spaces can be seen in HSA. Bleeding, necrosis and mitotic cells are common (Zachary 2017). Their appearance can be divide into capillary, cavernous or solid appearances (Patten *et al.* 2016).

The prognosis is poor for HSA. The tumor stages can be divided into 1-3. In stage 1, HSA is located int the primary organ, in stage 2 the primary HSA has ruptured or spread to the regional lymph node, and finally in the stage 3, more distant metastases are found. Dogs with stage 1 HSA has a longer median survival time (196 days) compared to stage 3 (23 days) (Batschinski *et al.* 2018). Studies have shown that with treatment with surgery alone, the median survival time is about 2 months. In comparison, surgery followed by doxorubicin treatment has a median survival time of about 9 months (Batschinski *et al.* 2018). In other words, independently of the stage of HSA, the prognosis is poor. This can be compared to the most common benign splenic lesion hematoma, with an excellent prognosis with a median survival time of 650 days post splenectomy (Patten *et al.* 2016).

2.2. Diagnostic parameters for splenic masses in dogs

The distinction between malignant and benign splenic masses is important due to the poor prognosis of HSA. The most common diagnostic methods are clinical features (Nelson & Couto 2019), imaging (Mallinckrodt & Gottfried 2011), fine

needle aspirate (O'Keefe & Couto 1987), cytology & biopsies (Nelson & Couto 2019) and hematology (Ng & Mills 1985; Johnson *et al.* 1989; Thamm *et al.* 2012).

The clinical features vary due to the underlying cause. Everything from diffuse symptoms such as vomiting, weight loss and inappetence, to spontaneous bleeding (petechia and ecchymoses), pale mucous membranes, hemoabdomen and collapse can be seen. Abdominal palpation may give suspicion of an enlarged spleen (Nelson & Couto 2019). Imaging such as radiographs and ultrasound may be able to detect splenic masses. This may also be useful in investigating potential metastases, or to determine the accurate size of the splenic lesion. However, due to the variation of appearance on imaging, it might be challenging to discriminate benign changes from malign (Mallinckrodt & Gottfried 2011; Schick *et al.* 2019). To gain cytological information, fine needle aspiration and needle biopsy can be obtained. This can be especially helpful in diagnosing neoplastic changes (O'Keefe & Couto 1987; Nelson & Couto 2019). However, as HSA often are surrounded by hematomas, as well as due to the challenge to exfoliating neoplastic cells into the sample, a definite diagnosis might not be determined. Hematology may also be used to distinguish between malign and benign masses. Some studies suggest that anemia, thrombocytepenia, schistocytosis, acanthocytosis and nucleated blood cells, are associated with malignant splenic neoplasia (Nelson & Couto 2019). However, some other studies discuss that abnormal hemostatic and hematologic results has no significant correlation with malignant or benign splenic tumor (Lee *et al.* 2018). To conclude, studies suggest that it is challenging to reach a definite diagnosis presurgically.

2.3. Biomarkers

According to the National Cancer Institute (NCI), a biomarker is "A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease". In veterinary oncology, potential applications of biomarkers include diagnosis, staging, prognosis and monitoring responses to treatment. Biomarkers can be assessed from, for example, urine, serum and tissue. However, as of today, few biomarkers have been evaluated and successfully implemented as a diagnostic method for HSA (Henry 2010; Thamm *et al.* 2012). Hence, there is a need to develop suitable biomarkers for supporting or dismissing the suspicion of HSA.

2.3.1. Proteoglycans

Glycosaminoglycans (GAGs) are present in every mammalian tissue and are sulfated, linear, negatively charged polysaccharides. Studies have shown that glycosaminoglycans affect cell properties and functions, interacting with growth

factors and acting directly on cell receptors. Hence, the altered structure of GAGs indicates their importance as biomarkers for disease diagnosis and progression, as well as pharmacological targets (Afratis *et al.* 2012).

Proteoglycans consists of a core protein linked with one or several glycosaminoglycan chains attached, creating complex molecules with diverse functional and structural properties (Korpetinou *et al.* 2014; Manou *et al.* 2020). Proteoglycans can be divided into three main groups depending on their locations: extracellular matrix proteoglycans, cell-surface associated proteoglycans and intracellular proteoglycans. They are synthesized by all cells and distributed in all tissues. They participate in physiological functions as well as pathologic conditions (Schaefer & Schaefer 2010; Theocharis *et al.* 2010; Korpetinou *et al.* 2014).

The proteoglycan expression in the tumor microenvironment has been discussed in several studies (Barkovskaya *et al.* 2020; Gubbiotti *et al.* 2020). In the context of malignant transformation, the type and fine structure of GAG chains attached to proteoglycans are affected. These structural modifications may facilitate tumorigenesis in various ways and hence changing the functions of proteoglycans (Korpetinou *et al.* 2014; Barkovskaya *et al.* 2020). Proteoglycans modulate cell signaling, tumor cell-matrix interactions and angiogenesis, either blocking disease progression or creating a favorable microenvironment for tumor cell growth and spreading (Manou *et al.* 2020).

2.3.2. Serglycin

Serglycin is categorized as an intracellular proteoglycan, even though numerous studies have shown that it is secreted by various cell types and incorporated into the extracellular matrix or attached to the cell surface (Scully *et al.* 2012; Korpetinou *et al.* 2014; Manou *et al.* 2020). The core protein is 17,6 kDa and contain a 16-amino acid serine/glycine repeat region to which GAGs are attached (Theocharis *et al.* 2010). Heparan sulfate, heparin, and chondroitin sulfate are glycosaminoglycans that may be attached to serglycin (Kolset & Tveit 2008). Due to the constant expression of serglycin in all normal and malignant hematopoietic cells, serglycin was initially considered a hematopoietic proteoglycan. In recent studies, it has been found that serglycin is also expressed by several non-hematopoietic cells such as smooth muscle cells, endothelial cells and chondrocytes (Kulseth *et al.* 1999; Korpetinou *et al.* 2014). It is also expressed in various tumor cells, especially in more aggressive cancer cells (Korpetinou et al. 2014). Lastly, it has shown response to infection and blood coagulation (Kolset & Pejler 2011), and was shown to have an important role in platelet functions (Woulfe *et al.* 2008).

Serglycin and inflammation

Serglycin has an important role in inflammatory reactions. More specifically, serglycin is expressed and essential to the functions of cytotoxic T-lymphocytes, mast cells, monocytes and neutrophils (Scully *et al.* 2012). In mast cells, neutrophils and cytotoxic T cells, serglycin is actively involved in synthesis and granula storage of inflammatory mediators (Åbrink *et al.* 2004; Kolset & Tveit 2008). Macrophages and monocytes normally don't contain granula. Instead, there is a low constitutive secretion of serglycin that increases with inflammation (Kolset & Tveit 2008). Serglycin is hence important for the key inflammatory mediators inside storage granules and secretory vesicles. When mice lacking serglycin were infected with lymphocytic choriomeningitis viruses, the contraction of the antiviral CD8+ T cell response was markedly delayed compared to serglycin-competent mice (Grujic *et al.* 2008). In another study, major neutrophil granule proteins in serglycin knockout mice were studied, where they found that neutrophil elastase was absent from mature neutrophils. When injected with Gram-negative bacteria (*Klebsiella pneumoniae*), the virulence was increased in the serglycin knockout mice compared with wild-type mice (Niemann *et al.* 2007). Despite this, serglycin does not seem to be absolutely necessary to fight infection (Scully *et al.* 2012).

Very few studies related to serglycin has been made on dogs. One study examined the upregulation of proteolysis-related genes in canine uterine bacterial infection. They concluded that serglycin was one of the genes showing the largest extent on upregulation in diseased uteri, supporting the previous similar studies made in mice (Hagman *et al.* 2009).

Serglycin and cancer

It is shown that serglycin is expressed in increased amounts in many tumor cells compared to healthy cells in humans. Also, more aggressive cancer has shown to express higher amounts of serglycin compared to less aggressive cancer forms in giant cell tumor of bone, lung cancer, colon cancer, prostate cancer, breast cancer, multiple myeloma and esophageal cancer (Skliris *et al.* 2011; Korpetinou *et al.* 2015; Theocharis *et al.* 2015; He *et al.* 2021; Zhu *et al.* 2021). Its involvement in the development of cancer has been discussed by several studies, such as affecting the tumor microenvironment, its inhibition of the complement system and its effect on cell proliferation and cell migration.

Tumor microenvironment plays an important role in the formation and progression of cancer. It is shown that serglycin is secreted by cancer cells, as well as the stroma cells associated with cancer, and that it further binds to the glycoprotein CD44 through the chondroitin-4 sulfate side chains (Guo *et al.* 2017; Zhu *et al.* 2021). CD44 is a transmembrane glycoprotein, and when activated in cancer cells, pro-

motes stemness, increases invasion and integration, and drives sphere formation (Skandalis *et al.* 2019). A knock-down of serglycin leads to a decreased number of cells with high CD44 levels, and hence serglycin promotes CD44 expression completing a positive feedback-loop activating tumor growth (Guo *et al.* 2017; Zhang *et al.* 2020).

Complement activation is an important part of the congenital immune system, and serglycin has shown to affect this activation. When looking at multiple myeloma as well as breast cancer, serglycin has shown inhibitory properties related to the complement system (Skliris *et al.* 2011; Korpetinou et al. 2013). The studies argue that this might protect the cancer cells and promote survival of malignant cells. Furthermore, the study examined the *in situ* expression of serglycin in breast cancer. The study showed that induced ectopic expression of serglycin in less aggressive breast cancer cells induced their proliferation and migration (Korpetinou *et al.* 2013). Similar findings were obtained when studying serglycin in nasopharyngeal carcinoma. When blocking serglycin expression in nasopharyngeal carcinoma cell lines *in vivo,* the metastatic capacity was reduced. Conversely, overexpression of serglycin in poorly metastatic cells increased their metastatic and motile behavior. Lastly, they found an upregulation of the serglycin expression in the liver metastases compared to the primary tumor (Li *et al.* 2011).

As of today, very few studies has been done related to serglycin and tumor development in dogs.

2.3.3. Eukaryotic elongation factor 2 (EEF2)

EEF2 is a protein that plays an essential role in the GTP-dependent translocation of the ribosome along mRNA, which is essential for protein synthesis. Protein translation can be divided into three phases: initiation, elongation and termination. EEF2 is hence essential in the elongation phase. EEF2 has been discussed both in terms of a reference gene as well as a biomarker in cancer (Bose & Sarma 1975; Eissa *et al.* 2017; Zhang *et al.* 2018). For example, one study looked at the stability of 13 reference gene expression in mice treated with DNBS- induced colitis. They concluded that EEF2 was one of the five most stable genes in the study (Eissa *et al.* 2017). However, especially in recent studies, EEF2 has been highlighted as a potential biomarker in cancer development. As one characteristic of cancer metabolism is high level of protein biosynthesis, EEF2s involvement in the elongation phase affects the development of cancer. The deregulation of the elongation step in translation has shown to be involved in tumorigenesis (Grzmil & Hemmings 2012; Shi *et al.* 2018; Zhang *et al.* 2018). Previous studies has, for example, shown an overexpression of EEF2 in lung, esophageal, pancreatic, breast and prostate cancers, HNSCC and glioblastoma multiforme (Oji *et al.* 2014; Pott *et al.* 2017).

As for serglycin, very few studies on EEF2 related to tumor development have been made on dogs as of today.

3. Material and methods

3.1. Selection of samples

Tumor samples with histopathological diagnosis, as well as non-pathological spleens, were obtained from the Department of Pathology (BVF, SLU) in the form of formalin-fixed and paraffin-embedded preparations (FFPE samples). To choose suitable samples, the samples were evaluated histologically. Only HSA samples with cavernous and solid appearances, and with neoplastic endothelial cells wrapped around stroma forming poorly defined blood-filled vascular spaces, were included. Samples with excessive amount of blood where not included. Furthermore, 7 samples from histopathologically confirmed non-pathological spleens were obtained.

3.2. Selection of genes analyzed

Reference genes were included to be used as controls to normalize the data, for example by correcting for differences in quantities of cDNA. Hence, optimal reference genes are those that does not exhibit changes in expression between samples from various time points and conditions, such as pathological conditions (bio-rad 2021). In this study, GAPDH, RPS5 and HPRT were chosen based on previous studies of rats and canine gene expressions (Brinkhof *et al.* 2006; Peters *et al.* 2007; Boerkamp *et al.* 2013; Košuth *et al.* 2019). EEF2 was also included in the study as a gene to compare the expression of serglycin.

3.3. Analysis of serglycin

3.3.1. Isolation of RNA

As the samples were obtained from paraffin embedded materials, the process started with removal of the fixation with paraffin according to the protocol described by appendix 1. Two to three incisions of 20 μm were made from each paraffin block.

To digest the paraffin, 100% Xylene was used in two batches. The sample was then washed twice with 99,5% ethanol, and proteinase K was added to degrade proteins and tissue structures before RNA was isolated. RNA from the samples was isolated according to a modified protocol with TRIzol Reagent (see Appendix 1 & 2). Chloroform was added to separate DNA and protein from RNA. The aqueous phase containing the RNA was transferred to a new tube. For precipitation of RNA, isopropanol was used. The RNA pellet samples were washed with 70% ethanol, allowed to air dry, and resuspended in 30 microliter RNAse-free water and then stored at -80 °C. Two microliter of the RNA sample was diluted with 3 µl Tris, and the RNA concentration was measured in all samples spectrophotometrically with NanoDrop to assess the amout of RNA.

3.3.2. Synthesis of cDNA

For synthesis of cDNA from isolated RNA, a protocol was followed with Super-script IV and DNAse II Invitrogen AMPD1 (see Appendix 3). The primers used were equal parts Oligo dT as well as reverse primers for the reference genes and serglycin and EFF2. For each sample, a negative control was also made with samples without "reverse transcriptase".

3.3.3. Analysis with qPCR

The gene expression of serglycin and EEF2 was measured by qPCR according to the protocol in Appendix 4. The cDNA to be analyzed was diluted with RNAse-free water to 1: 5. In this study, three reference genes (RPS5, GAPDH and HPRT) were used. Three tubes were prepared with "forward" and "reverse primers" for each gene, together with Cybergreen PCR mix and nuclease free water. All samples where then measured for all reference genes as well as Serglycin and EEF2. For each sample, a negative control (reverse transcriptase negative, RT-) was also performed with samples not containing any cDNA in the qPCR analysis and no genes should then be measurable. To control contamination and dimer formation of primers, non-template controls were also performed with RNAse-free water instead of cDNA, as well as non-templete controls with only the PCR mix. To include the sample in the study, the result needed to express the correct melting point for the specific gene and show no other peaks above 600-d(RFU)/dt (for example significant contaminations). If only one of the two duplicates showed the right expression, only the correct one was included. Lastly, the Cq values had to be under 35,5. Though these criteria, only samples showing the correct expression for the specific gene were included in the study.

3.3.4. Statistics

To compare the expression of Serglycin and EEF2 in HSA samples with the healthy spleen samples, the double delta Ct analysis (Livak) method was used ("Real-Time PCR Experimental Design | LSR | Bio-Rad," n.d.). The reason for this is that the values obtained from the qPCR cannot be directly compared between the healthy tissue and the tissue including HSA, as the differences in how much gene expression each tissue generates in total must be considered. Through the calculation of the livak method, where the reference genes also are included, a ratio called fold change is obtained. This fold change can be compared with that for normal tissue, which has a value of 1.0. A value above 1.0 thus means that the tissue expresses more serglycin than normal. As the control group of samples of healthy spleens were gathered from different individuals and from a different number of individuals than the ones with HSA, the reference sample used was the "geometric average" of the mean of the two reference genes to compensate for variable values. Student t-test with Welsh correction in the program GraphPad Prism 9 was used to calculate significance and p-value.

4. Results

4.1. Isolation of RNA

To assess whether the isolation of RNA was successful or not, the absorbance values for all samples were analyzed in NanoDrop. The RNA concentration varied between 193 ng/µl and 1537 ng/µl in the HSA FFPE samples, and between 29,1 ng/µl and 93 ng/µl in the non-pathological spleen FFPE samples. A260/280 and A260/230 ratios are both measures to evaluate the amount of contamination in the sample. A value of approximately 1,8-2,1 for A260/280 and about 2,0 for A260/230 is counted as "pure" for RNA. Through values below 2 on the ratio 260/230, the majority of the samples show signs of contamination in the form of organic substances such as TRIzol. For samples including HSA, almost all samples were between 1,8-2,1 in the ratio 260/280, indicating little contamination of proteins (Table 1). However, for the non-pathologic spleen samples, all samples show contamination of proteins as the values lies between 1,44 and 1,54 in the ratio 260/280.

Chart 1. Results from NanoDrop measurement of RNA concentration in FFPE samples

Individual/FFPE-sample	Concentration (ng/µl)	260/280	260/230
HSA 1	754,8	1,84	0,64
HSA 2	404,8	1,84	0,56
HSA 3	1537,5	1,97	0,94
HSA 4	451,0	1,82	0,45
HSA 5	848,3	1,79	0,73
HSA 6	399,8	1,82	0,45
HSA 7	277,3	1,81	0,29
HSA 8	998,8	1,84	1,04
HSA 9	193,9	1,89	0,25
HSA 10	948,0	1,81	0,8
HSA11	675,0	1,81	0,58
HSA 12	956,3	1,83	0,81
HSA 13	651,5	1,91	0,65

HSA 14	795,3	1,76	0,7
HSA 15	894,8	1,84	0,77
HSA 16	641,0	1,82	0,55
HSA 17	593,8	1,79	0,54
HSA 18	457,0	1,82	0,48
HSA 19	563,3	1,73	0,5
HSA 20	669,0	1,79	0,63
Healthy spleen 1	55,5	1,54	0,26
Healthy spleen 2	68,3	1,48	0,23
Healthy spleen 3	29,1	1,52	0,28
Healthy spleen 4	63,1	1,47	0,31
Healthy spleen 5	54,5	1,54	0,31
Healthy spleen 6	93,5	1,71	0,57
Healthy spleen 7	42,6	1,44	0,26

4.2. Synthesis of cDNA

Prior the synthesis of cDNA, a PCR and gel electrophoresis was mad on living cell culture from mastocytoma for all the different reference genes as well as serglycin and EEF2 to see if the primers worked. The gel showed clear PCR products of the expected size for both control genes and serglycin, without primer dimers or other PCR products. This indicates that the primer design for qPCR of the genes is good. After the cDNA synthesis, all samples and genes were tested for qPCR to see if the protocol and synthesis had worked. HPRT showed wrong expression with multiple tops and melting points for almost all samples. Hence gel electrophoresis was made on 2 samples (6, 12), where products of the wrong size could be seen. Therefore, HPRT were excluded from the study. The other genes showed correct melting points and expressions for the majority of the genes and samples and were hence included in the study.

4.2.1. Serglycin

The results from the qPCR and fold change for serglycin are shown in chart 2. Several samples showed incorrect signals that didn't fulfill the criteria discussed in the method and was hence excluded from the study. This means that the calculation of fold change could not be performed for all samples. Nine out of 20 samples gave correct signals for HSA and serglycin, and 6 out of 7 samples gave corrects signals for non-pathological spleens and serglycin. Fold change was calculated and an expression between 0,4 and 14,7 times was seen for HSA and serglycin.

Chart 2. Results from qPCR SGN, mean value of Cq for the duplicates and fold change for HAS and non-pathological spleens

FFPE-sample	SGN	GAPDH	RPS5	Fold change
HSA 1	-	34,3	32,1	-
HSA 2	31,3	35,2	25,5	4,0
HSA 3	29,9	27,1	25,4	10,6
HSA 4	29,9	29,6	27,0	10,9
HSA 5	-	32,3	-	-
HSA 6	-	31,2	-	-
HSA 7	-	31,1	-	-
HSA 8	-	30,4	-	-
HSA 9	32,0	30,0	28,0	2,4
HSA 10	-	32,0	-	-
HSA11	31,4	26,9	27,1	3,9
HSA 12	-	31,3	30,4	-
HSA 13	29,4	26,1	25,2	14,7
HSA 14	34,6	-	-	0,4
HSA 15	33,8	29,6	28,6	0,7
HSA 16	-	31,9	-	-
HSA 17	-	31,6	-	-
HSA 18	31,9	28,3	26,2	2,7
HSA 19	-	-	-	-
HSA 20	-	33,7	32,7	-
Healthy spleen 1	32,7	29,3	29,0	1,3
Healthy spleen 2	33,7	29,4	28,3	0,6
Healthy spleen 3	35,3	30,5	29,2	0,2
Healthy spleen 4	33,6	-	-	0,7
Healthy spleen 5	31,0	27,5	27,6	4,3
Healthy spleen 6	-	-	-	-
Healthy spleen 7	32,2	30,4	30,0	1,9

4.2.2. EEF2

EEF2 and its fold change are shown in chart 3. As for serglycin, several samples showed incorrect signals that didn't fulfill the criteria discussed in the method and was hence excluded from the chart and the study. For EEF2 11 out of 20 samples showed correct expression for HSA, and 5 out of 7 samples showed correct expression for the non-pathological spleens. The fold change was between 0,1 and 23,4.

Chart 3. Results from qPCR EEF2, mean value of Cq for the duplicates and fold change for HAS and non-pathological spleens

FFPE-sample	EEF2	GAPDH	RPS5	Fold change
HSA 1	-	34,3	32,1	-
HSA 2	29,2	35,2	25,5	3,4
HSA 3	28,3	27,1	25,4	6,4
HSA 4	29,4	29,6	27,0	2,9
HSA 5	-	32,3	-	-
HSA 6	-	31,2	-	-
HSA 7	-	31,1	-	-
HSA 8	-	30,4	-	-
HSA 9	31,2	30,0	28,0	0,9
HSA 10	-	32,0	-	-
HSA11	28,5	26,9	27,1	5,5
HSA 12	33,3	31,3	30,4	0,2
HSA 13	26,4	26,1	25,2	23,4
HSA 14	-	-	-	-
HSA 15	33,1	29,6	28,6	0,2
HSA 16	-	31,9	-	-
HSA 17	33,7	31,6	-	0,1
HSA 18	28,2	28,3	26,2	6,8
HSA 19	-	-	-	-
HSA 20	34,2	33,7	32,7	0,1
Healthy spleen 1	30,8	29,3	29,0	0,9
Healthy spleen 2	30,8	29,4	28,3	0,9
Healthy spleen 3	31,3	30,5	29,2	0,7
Healthy spleen 4	-	-	-	-
Healthy spleen 5	29,0	27,5	27,6	3,2
Healthy spleen 6	-	-	-	-
Healthy spleen 7	31,6	30,4	30,0	0,5

4.3. Fold change

Fold change was compared between the samples including HSA and the samples including non-pathological spleens for both serglycin and EEF2. They were also tested statistically with Welch t-test and descriptive statistics in GraphPad Prism 9.

4.3.1. Serglycin

As seen in figure 1, there was a significant difference in fold change between the samples including HSA and non-pathological spleens (p-value 0,048). The samples including HSA had a mean value of 5,6 with a “standard deviation error of mean” of 1,7 for serglycin. The samples including non-pathological spleens had a mean value of 1,5 with a “standard deviation error of mean” of 0,6.

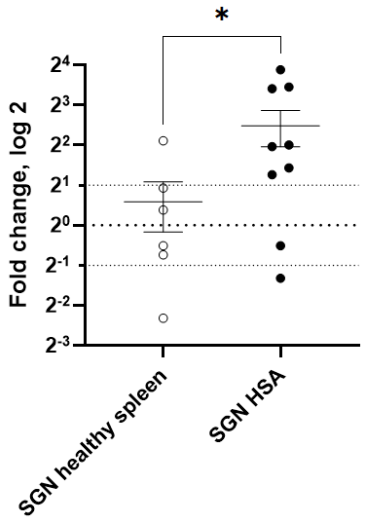

Figure 1. Fold change for serglycin from HSA and non-pathological spleens.

4.3.2. EEF2

The fold change for EEF2 is shown in figure 2. Unlike serglycin, there was no significant difference when comparing samples including HSA and non-pathological spleens (p-value 0,145). The HSA samples had a mean value of 4,5 with a “standard deviation error of mean” of 2,0, and the non-pathological samples had a mean value of 1,2 with a “standard deviation error of mean” of 0,5.

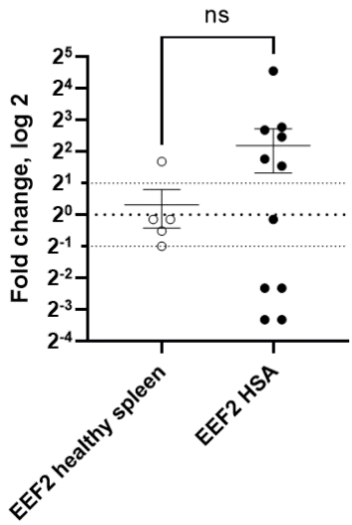

Figure 2. Fold change for EEF2 from HSA and non-pathological spleens.

5. Discussion

5.1. Isolation of the RNA

RNA extraction from all FFPE samples generated measurable levels of RNA during analysis in NanoDrop. However, with great variation and clear signs of contamination. One potential explanation for the contamination could be the method where TRIzol was used. One study compared three different RNA extraction methods, where TRIzol was one of them (Kim *et al.* 2014). The two other ones were the PAXgene and NucleoSpin kits. They found that TRIzol gave a higher degree of contamination compared to the two other methods, but also that RNA concentrations were higher with the TRIzol method. Another explanation for the contamination when using TRIzol is the step when pipetting away the supernatant containing RNA from the other layers. As the interphase and bottom layer contains organic substances such as DNA and proteins, there is a risk that these are included as well (Grabmüller *et al.* 2015). Lastly, proteins and DNA are also possible contaminants in the RNA extraction. To reduce remained protein and DNA, an increased number of washes could potentially reduce the contamination.

There was a significant variation in the concentration between the samples including HSA and the samples from non-pathological spleens. The non-pathological spleens showed clearly lower concentrations. One explanation could be that HSA in the spleen includes more cells than non-pathological spleens. Another explanation could be the step where the supernatant is to be pipetted away without disturbing the pellet with RNA. The samples including HSA showed a clear pellet, when again no clear pellet was seen in the samples with non-pathological spleens. One study, made on blood, stated that that RNA is often partially, or completely, lost in this step (Eikmans *et al.* 2013).

5.2. The Cq values

The quality of the qPCR results from the FFPE samples varied. This makes the results unreliable and difficult to draw conclusions about. 11 samples including

HSA did not fulfill the criteria's for serglycin, and hence did not express correct and trustable values to be analyzed. These values have therefore not been included in the calculations. The most likely reasons why these samples and genes could not be measured are failed synthesis of cDNA, improper pipetting, contaminations, or degenerated quality of the FFPE sample. Since the qPCR mix, the water and the negative controls (RT-) were all negative in the qPCR results, it is not likely that the issue lays in these products or is due to contamination. For serglycin, 4 HSA samples completely lacked values and some individuals gave only single values instead of the duplicates analyzed. Also, 6 samples gave high Cq values, which can occur when the method loses sensitivity or when the amount of genetic material analyzed is too small. Hence, one logical explanation could be improper pipetting. As the concentrations were clearly higher for the HSA samples than for the non-pathological samples, very little amounts were taken from the HSA samples when diluting all samples to reach the same concentration prior to the cDNA synthesis. In this stage, too little RNA might have been obtained when pipetting. A failed cDNA synthesis could also be a possible explanation, but since it was repeated for some of the samples without any improvement in results, it is less likely.

When it comes to the reference genes and EEF2, the most common lack was the wrong melting point and several peaks, hence not fulfilling the criteria's. This could have been a result of failed synthesis of the cDNA, not properly working primers or just deteriorated material from the FFPE samples. Also, as the non-pathological samples worked well for almost all genes and samples, the problem might also be the HSA, making the analysis more challenging compared to the healthy tissue. Hence, it could be valuable to compare another method for FFPE HSA samples.

5.3. Serglycin as a biomarker

HSA is a common tumor in the spleen in dogs and has a poor prognosis (Patten *et al.* 2016; Batschinski *et al.* 2018), when again benign masses such as hematoma has a good prognosis (Spangler & Kass 1997). As of today, there is a lack of more detailed diagnostic parameters to distinguish between the malign and benign masses before splenectomy (Mallinckrodt & Gottfried 2011; Nelson & Couto 2019). In this study, serglycin expression in HSA tissue were compared with serglycin expression in non-pathological spleen tissue in FFPE samples. EEF2 was also included to be compared with serglycin. All the samples came from different individuals and had been stored for a different amount of time. The oldest sample was from 2012. The non-pathological samples were all from 2021. The HSA samples had all been diagnosed trough histology and showed high amounts of tumor cells. Also, seventeen out of twenty samples had had a ruptured spleen. Out of the 20 individuals, the

most common races were Labrador Retriever, Flat-Coated Retriever and Shepherd. There was no clear correlation between the level of mitotic activity, or other histological findings, in the tumor and the expression of serglycin.

Fold change was above 1,0 for the majority of the HSA samples, which means that serglycin expression were higher in these than in the samples including non-pathological spleen tissue. A significant difference in serglycin expression was seen between HSA and the non-pathological samples. This is in line with previous studies in humans when studying serglycin expression in tumor tissue compared to healthy tissue (Skliris *et al.* 2011; Korpetinou *et al.* 2015; Theocharis *et al.* 2015; He *et al.* 2021; Zhu *et al.* 2021). Another aspect of this study is that it compared HSA to healthy tissue in the spleen. It would also be interesting and valuable to compare HSA to other masses in the spleen, such as hematoma. However, as very few studies related to serglycin expression in tumors has been made on dogs, the finding in this study is interesting and could encourage further research within the area.

When looking at EEF2, EEF2 showed no significant difference when comparing the samples including HSA with samples including healthy tissue from spleen. Also, the standard deviation as well as the standard deviation error of mean was higher for EEF2 than with serglycin for samples including HSA. Hence, this study suggests serglycin to be more suitable as a potential biomarker for HSA than EEF2.

Even though the quality of the qPCR results for the FFPE samples varied, which makes the results difficult to draw conclusions from, this thesis still shows that there are trends that are interesting to continue to investigate with other methods and a larger patient material as a basis. Based on the samples showing the correct expression, this study support that a correlation between the expression of serglycin and cancer in FFPE samples. However, to be used in practice as a diagnostic method, an easy and cheap way to analyze the expression is needed. Both FNA as well as blood samples would be valuable as indicators of HSA, as there are few diagnostic parameters giving a more detailed diagnosis as of today (Mallinckrodt & Gottfried 2011; Nelson & Couto 2019). Furthermore, more studies are needed on dogs to gain more knowledge regarding the serglycin expression from HSA and normal variation from healthy spleens. Despite some deficient results, serglycin seems to be increasingly expressed in tumor tissue and is worth continuing to study as a potential biomarker for HSA in dogs.

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Popular science summary

Introduction

Hemangiosarcoma (HSA) is a common cancer in the spleen in dogs. The prognosis is poor for HSA, usually with a survival time of under one year. Today, many dogs are euthanized due to suspicion of HSA in the spleen as the operation is costly and has a poor prognosis. However, the prognosis is good for many other changes is the spleen. With today's diagnostic methods, it is challenging to distinguish HSA from other masses in the spleen. With better diagnostic methods, several of these dogs could be cured as the tentative diagnosis may not be correct.

A new and hopefully possible way to diagnose cancer in dogs is to measure the levels of serglycin. Serglycin is a proteoglycan, which is a structure made up of proteins and carbohydrates, that is mainly expressed by immune cells. Recent studies in human cancer have shown a correlation between increased expression of serglycin from tumor cells and cancer. Hence, a quantification of serglycin in dogs could thus indicate HSA and be used for diagnostic purposes. The purpose of this study was to compare the serglycin expression in HSA to healthy tissue from the spleen. Another gene, EEF2, was also included in other to be compared with the expression of serglycin.

Literature review

Masses in the spleen

Masses in the spleen can be divided into two categories: cancer and other masses. Studies have shown, that in dogs with masses in the spleen, approximately 50% are cancer, and about 90% of these are malignant. Of the malignancies, HSA is the most common one. The prognosis differs strongly between the splenic masses, where HSA has a clearly lower survival time.

HSA is a common type of cancer in the spleen in dogs and can originate in principle wherever blood vessels are found, for example in the spleen, liver, intestines, heart and lungs. The tumor has an aggressive character and can infiltrate and spread to other organs. The clinical symptoms of HSA are mostly due to the secondary effects of the tumor, such as tumor growth or rupture, and it can result in an animal in usually very poor condition. Removal of the spleen is recommended as a treatment

due to the risk of rupture. The prognosis is poor for HAS with usually a survival time of under 12 months.

Diagnostic parameters for splenic masses in dogs

The distinction between malignant and other masses is important due to the poor prognosis for HSA. As of today, HSA is usually diagnosed with the state of the animal, x-rays, ultrasound, and other methods such as blood samples. However, it is challenging to reach a definite diagnosis with these methods.

Biomarkers

A biomarker is a biological molecule in the body that can, for example, be used as a diagnostic parameter for diseases when quantified. However, few biomarkers have been evaluated and successfully implemented as a diagnostic method for HSA as of today. Hence, there is a need to develop suitable biomarkers for supporting or dismissing the suspicion of HSA.

Proteoglycans

Proteoglycans are structures made up of proteins and carbohydrates that are found inside and outside the cells. Most proteoglycans are located inside the cells where they contribute to the signal transmission that gives the cell its functions. This means that proteoglycans are generally involved in various reactions in the body, such as inflammation. The proteoglycan expression has also shown to be affected in cancer.

Serglycin

Serglycin is a proteoglycan and is expressed by various cells in the body and is mostly involved in the immune system. It has also been shown that it is expressed in various cancer cells. When it comes to inflammation, serglycin affects how the so-called granules, that includes chemical substances, are stored in the immune cells. When released, these chemical substances cause reactions that are seen in inflammation. When serglycin is not present, the body's ability to defend itself against infections, injuries and diseases is affected. In addition, studies have shown that serglycin is expressed in increased amounts in cancer cells compared to healthy cells in humans. More aggressive cancer has shown to express higher amounts of serglycin compared to less aggressive cancer forms. Serglycin seems to be involved in both tumor growth and the spread of cancer. As of today, very few studies has been done related to serglycin and tumor development in dogs.

Eukaryotic elongation factor 2 (EEF2)

EEF2 is a protein that plays an essential role in the production of proteins in the cells. As cancer is dependent of the level of protein metabolism, EEF2 has also been described as a potential biomarker in cancer development. Previous studies in human cancer have shown an overexpression of EEF2 in cancer. As for serglycin, very few studies on EEF2 related to tumor development have been made on dogs.

Material, methods and the statistics

In this study, the expression of serglycin from tumor cells in dogs has been measured and compared with normal splenic tissue. Another gene, EEF2, was also included in the study to be compared with the expression of serglycin. The HSA tumor samples, which were fixed in formalin and embedded in paraffin in order to store the material for a longer period of time, were collected from the Department of Pathology at the Swedish University of Agricultural Sciences. The selection of the samples was made through histological examination, that is though, looking at the sample and its cells through a microscope. In addition to the genes serglycin and EEF2, 2 more genes were analyzed. These were so called reference genes, that shouldn't vary too much in cancer tissue and could hence be used when analyzing the results.

To compare the expression of serglycin in tumors with normal tissue, a statistical method called the Livak method was used. The reason for this is that the values obtained from qPCR cannot be compared directly as the tumor samples and healthy samples were taken from different individuals and from a different number of individuals. Results from these analyzes have then been statistically processed to see if there is any statistically significant difference between individuals with HSA and healthy dogs.

Results and discussion

The results from this study show that HSA tissue from dogs has a higher expression of serglycin than normal splenic tissue, which is in line with previous studies on human cancer. This was not the case for EEF2, indicating that serglycin would be a more suitable diagnostic biomarker for HSA than EEF2. Another aspect is that HSA was now compared with healthy spleens, but it would also be valuable to compare HSA with other splenic masses. Also, several samples were excluded from the study due to incorrect expressions, hence affecting the trustability of the study. To ensure the conclusion from this study, more tumor samples from dogs need to be analyzed and compared with normal tissue. Lastly, to be used in practice as a diagnostic method, an easy and cheap way to analyze the expression is needed. Despite some deficient results, serglycin seems to be increasingly expressed in tumor tissue and is worth continuing to study as a potential biomarker for HSA in dogs

Appendix

Appendix 1. Preparing total RNA from FFPE blocks

- Using a microtome cut two (or three slices if smaller amounts of tissues) slices 20 µm thick and transfer to 1,5-ml siliconized tubes

- Incubate the slices twice in 1 ml of xylene at 50°C for 5 minutes, followed by centrifugation at 13,000rpm

- Wash pellets twice in 1 ml of 99,5% ethanol and air-dry at room temperature. (If the pellets were not attached to the tubes surface, it was centrifugated at 13000rpm for 1 minute).

- Incubate the dried pellets in 150 µm of 1X proteinase K digestion buffer (20mmol/L Tris-HCl, pH 8.0; 1mmol/L CaCl2, 0,5% sodium dodecyl sulfate), containing 400 µm/ml proteinase K at 55°C for 3 hours. Incubate in 95°C for 5 minutes and cool down to room temperature.

- Extract total RNA using the 1 ml TRIzol (Invitrogen, Carlsbad, CA) method according to the manufacturer's instructions (please see appendix 2 for page 1-3 of the manufacturer's instructions). Some adaptions were made based on empirical evidence, and these adaptions are written in the specific step:

    - Add 0,2 mL of chloroform per 1 mL of TRIzol reagent used for lysis, then securely cap the tube
    - Incubate for 2-3 minutes. Adaptions: This step was changed. samples were incubated at least 10 minutes to increase the yield, and then the samples were vortexed every 2-3 minutes.
    - Centrifuge samples 15 minutes at 12,000 x g at 4°C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase.

    - Transfer the aqueous phase containing the RNA to a new tube. Adaption: About 500-600 µm was transferred from each sample.
    - Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45 °C and pipetting the solution out. IMPORTANT: avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

- Isolate RNA according to the manufacturer's instructions:

    - Add 0,5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol reagent used for lysis. Adaption: 500-600 µm of isopropanol was added.
    - Incubate for 10 minutes or more.
    - Centrifuge for 10 minutes at 12,000 x g at 4°C. Total RNA precipitate forms a white gel like pellet at the bottom of the tube.
    - Discard the supernatant with a micropipette.

- Wash the RNA according to the manufacturer's instructions:

    - Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol reagent used for lysis. Adaptions: ethanol of 70% was used instead of 75%. Note: the RNA can be stored in 75 % ethanol for at least 1 year at -20 °C, or at least one week at 4 °C.
    - Vortex the sample briefly, then centrifugate for 5 minutes at 7500 x g at 4°C.
    - Discard the supernatant with a micropipettor.
    - Vacuum or air dry the RNA pellets for 5-10 minutes.
    - 30 µL of nuclease free water was added after air drying, and then incubated in 55°C for 10 minutes.

- Determine the yield and quality 260/280 optical density (OD) ratios of RNA product by the nano-drop spectrophotometer

Appendix 2: TRIZOL, pages 1 to 3 of the manufacturer's instructions

invitrogen USER GUIDE

TRIzol™ Reagent

Catalog Numbers 15596026 and 15596018

Doc. Part No. 15596026.PPS **Pub. No.** MAN0001271 **Rev.** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Invitrogen™ TRIzol™ Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour. TRIzol™ Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. TRIzol™ Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. TRIzol™ Reagent allows for simultaneous processing of a large number of samples, and is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).

TRIzol™ Reagent allows to perform sequential precipitation of RNA, DNA, and proteins from a single sample (Chomczynski, 1993). After homogenizing the sample with TRIzol™ Reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol. DNA is precipitated from the interphase/organic layer with ethanol. Protein is precipitated from the phenol-ethanol supernatant by isopropanol precipitation. The precipitated RNA, DNA, or protein is washed to remove impurities, and then resuspended for use in downstream applications.

- Isolated RNA can be used in RT-PCR, Northern Blot analysis, Dot Blot hybridization, poly(A)+ selection, in vitro translation, RNase protection assay, and molecular cloning.
- Isolated DNA can be used in PCR, Restriction Enzyme digestion, and Southern Blots.
- Isolated protein can be used for Western Blots, recovery of some enzymatic activity, and some immunoprecipitation.

For DNA isolation, see the *TRIzol™ Reagent (DNA isolation) User Guide* (Pub. No. MAN0016385).

TRIzol™ Reagent can also be used with Phasemaker™ Tubes (Cat. No. A33248) to isolate RNA. Phasemaker™ Tubes creates a solid barrier between the organic and aqueous phases of the TRIzol™ Reagent following sample homogenization which makes separation of phases easier. See the *TRIzol™ Reagent and Phasemaker™ Tubes Complete System User Guide* (Pub. No. MAN0016163) for the full protocol.

TRIzol™ Reagent can also be used with the PureLink™ RNA Mini Kit (Cat. No. 12183018A) which uses spin columns instead of ethanol precipitation to purify the RNA. For additional information, see the *PureLink™ RNA Mini Kit User Guide* (Pub. No. MAN0000406).

Contents and storage

Contents	Cat. No. 15596026 (100 reactions)	Cat. No. 15596018 (200 reactions)	Storage
TRIzol™ Reagent	100 mL	200 mL	15–30°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 1 Materials required for all isolations

Item	Source
Equipment	
Centrifuge and rotor capable of reaching 12,000 × *g* and 4°C	MLS
Tubes	
Polypropylene microcentrifuge tubes	MLS
Reagents	
Chloroform	MLS

Table 2 Materials required for RNA isolation

Item	Source
Equipment	
Water bath or heat block at 55–60°C	MLS
Reagents	
Isopropanol	MLS
Ethanol, 75%	MLS
RNase-free water or 0.5% SDS	MLS
(Optional) RNase-free glycogen	MLS

Table 3 Materials required for protein isolation

Item	Source
Equipment	
(Optional) Dialysis membranes	MLS
Reagents	
Isopropanol	MLS
Ethanol, 100%	MLS
0.3 M Guanidine hydrochloride in 95% ethanol	MLS
1% SDS	MLS

Input sample requirements

IMPORTANT! Perform RNA isolation immediately after sample collection or quick-freeze samples immediately after collection and store at –80°C or in liquid nitrogen until RNA isolation.

Sample type	Starting material per 1 mL of TRIzol™ Reagent
Tissues[1]	50–100 mg of tissue
Cells grown in monolayer	1×10^5–1×10^7 cells grown in monolayer in a 3.5-cm culture dish (10 cm^2)
Cells grown in suspension	5–10 × 10^6 cells from animal, plant, or yeast origin or 1 × 10^7 cells of bacterial origin

[1] Fresh tissues or tissues stored in RNAlater™ Stabilization Solution (Cat. No. AM7020).

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Use cold TRIzol™ Reagent if the starting material contains high levels of RNase, such as spleen or pancreas samples.
- Use disposable, individually wrapped, sterile plastic ware and sterile, disposable RNase-free pipettes, pipette tips, and tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
- Always use proper microbiological aseptic techniques when working with RNA.
- Use RNaseZap™ RNase Decontamination Solution (Cat. no. AM9780) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes used during purification.
- Ensure that all materials that come into contact with TRIzol™ Reagent are compatible with phenol, guanidine isothiocyanate, and chloroform.

Lyse samples and separate phases

1. Lyse and homogenize samples in TRIzol™ Reagent according to your starting material.
   - **Tissues:**

     Add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample and homogenize using a homogenizer.
   - **Cell grown in monolayer:**
     a. Remove growth media.
     b. Add 0.3–0.4 mL of TRIzol™ Reagent per $1 \times 10^5 - 10^7$ cells directly to the culture dish to lyse the cells.
     c. Pipet the lysate up and down several times to homogenize.
   - **Cells grown in suspension:**
     a. Collect the cells by centrifugation and discard the supernatant.
     b. Add 0.75 mL of TRIzol™ Reagent per 0.25 mL of sample (5–10 × 10^6 cells from animal, plant, or yeast origin or 1 × 10^7 cells of bacterial origin) to the pellet.

        Note: Do not wash cells before addition of TRIzol™ Reagent to avoid mRNA degradation.
     c. Pipet the lysate up and down several times to homogenize.

   Note: The sample volume should not exceed 10% of the volume of TRIzol™ Reagent used for lysis.

   STOPPING POINT Samples can be stored at 4°C overnight or at –20°C for up to a year.
2. *(Optional)* If samples have a high fat content, centrifuge the lysate for 5 minutes at 12,000 × *g* at 4–10°C, then transfer the clear supernatant to a new tube.
3. Incubate for 5 minutes to allow complete dissociation of the nucleoproteins complex.
4. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, securely cap the tube, then thoroughly mix by shaking.
5. Incubate for 2–3 minutes.
6. Centrifuge the sample for 15 minutes at 12,000 × *g* at 4°C.

   The mixture separates into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase.
7. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.

   IMPORTANT! Avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

Proceed directly to "Isolate RNA" on page 2.

To isolate DNA or protein, save the interphase and organic phase. See the *TRIzol™ Reagent (DNA isolation) User Guide* (Pub. No. MAN0016385) or see "Isolate proteins" on page 3 for detailed procedures. The organic phase can be stored at 4°C overnight.

Isolate RNA

1 Precipitate the RNA

a. *(Optional)* If the starting sample is small (<10^6 cells or <10 mg of tissue), add 5–10 µg of RNase-free glycogen as a carrier to the aqueous phase.

   Note: The glycogen is co-precipitated with the RNA, but does not interfere with subsequent applications.
b. Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis.
c. Incubate for 10 minutes at 4°C.
d. Centrifuge for 10 minutes at 12,000 × *g* at 4°C.

   Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.
e. Discard the supernatant with a micropipettor.

2 Wash the RNA

a. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis.

   Note: The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.
b. Vortex the sample briefly, then centrifuge for 5 minutes at 7500 × *g* at 4°C.
c. Discard the supernatant with a micropipettor.
d. Vacuum or air dry the RNA pellet for 5–10 minutes.

   IMPORTANT! Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry, to ensure total solubilization of the RNA. Partially dissolved RNA samples have an $A_{230/260}$ ratio <1.6.

3 Solubilize the RNA

a. Resuspend the pellet in 20–50 µL of RNase-free water, 0.1 mM EDTA, or 0.5% SDS solution by pipetting up and down.

   IMPORTANT! Do not dissolve the RNA in 0.5% SDS if the RNA is to be used in subsequent enzymatic reactions.
b. Incubate in a water bath or heat block set at 55–60°C for 10–15 minutes.

Proceed to downstream applications, or store the RNA at –70°C.

4 Determine the RNA yield

Determine the RNA yield using one of the following methods.

Method	Procedure
Absorbance Absorbance at 260 nm provides total nucleic acid content, while absorbance at 280 nm determines sample purity. Since free nucleotides, RNA, ssDNA, and dsDNS absorb at 260 nm, they all contribute to the total absorbance of the sample.	1. Dilute sample in RNase-free water, then measure absorbance at 260 nm and 280 nm. 2. Calculate the RNA concentration using the formula A260 × dilution × 40 = µg RNA/mL. 3. Calculate the A260/A280 ratio. A ratio of ~2 is considered pure. RNA samples can be quantified by absorbance without prior dilution using the NanoDrop™ Spectophotometer. Refer to the instrument's instructions for more information.
Fluorescence Fluorescence selectively measures intact RNA, but does not measure protein or other contaminant present in the sample	• Quantify RNA yield using the appropriate Qubit™ or Quant-iT™ RNA Assay Kit (Cat. Nos. Q32852, Q10210, Q33140, or Q10213). Refer to the kit's instructions for more information.

Table 4 Typical RNA ($A_{260/280}$ of >1.8) yields from various starting materials

Starting material	Quantity	RNA yield
Epithelial cells	1×10^6 cells	8–15 µg
New tobacco leaf	—	73 µg
Fibroblasts	1×10^6 cells	5–7 µg
Skeletal muscles and brain	1 mg	1–1.5 µg
Placenta	1 mg	1–4 µg
Liver	1 mg	6–10 µg
Kidney	1 mg	3–4 µg

Isolate proteins

Isolate the proteins from the organic phase saved from "Isolate RNA" on page 2 using either "Precipitate the proteins" on page 3 or "Dialyse the proteins" on page 4.

1 Precipitate the proteins

a. Remove any remaining aqueous phase overlying the interphase.
b. Add 0.3 mL of 100% ethanol per 1 mL of TRIzol™ Reagent used for lysis.
c. Cap the tube, mix by inverting the tube several times.
d. Incubate for 2–3 minutes.
e. Centrifuge for 5 minutes at 2000 × *g* at 4°C to pellet the DNA.
f. Transfer the phenol-ethanol supernatant to a new tube.
g. Add 1.5 mL of isopropanol to the phenol-ethanol supernatant per 1 mL of TRIzol™ Reagent used for lysis.
h. Incubate for 10 minutes.
i. Centrifuge for 10 minutes at 12,000 × *g* at 4°C to pellet the proteins.
j. Discard the supernatant with a micropipettor.

2 Wash the proteins

a. Prepare a wash solution consisting of 0.3 M guanidine hydrochloride in 95% ethanol.
b. Resuspend the pellet in 2 mL of wash solution per 1 mL of TRIzol™ Reagent used for lysis.
c. Incubate for 20 minutes.

Note: The proteins can be stored in wash solution for at least 1 month at 4°C or for at least 1 year at –20°C.

d. Centrifuge for 5 minutes at 7500 × *g* at 4°C.
e. Discard the supernatant with a micropipettor.
f. Repeat step 2b–step 2e twice.
g. Add 2 mL of 100% ethanol, then mix by vortexing briefly.
h. Incubate for 20 minutes.
i. Centrifuge for 5 minutes at 7500 × *g* at 4°C.
j. Discard the supernatant with a micropipettor.
k. Air dry the protein pellet for 5–10 minutes.

IMPORTANT! Do not dry the pellet by vacuum centrifuge.

3 Solubilize the proteins

a. Resuspend the pellet in 200 µL of 1% SDS by pipetting up and down.

Note: To ensure complete resuspension of the pellet, we recommend that you incubate the sample at 50°C in a water bath or heat block.

b. Centrifuge for 10 minutes at 10,000 × *g* at 4°C to remove insoluble materials.
c. Transfer the supernatant to a new tube.

Measure protein concentration by Bradford assay (SDS concentration must be <0.1%), then proceed directly to downstream applications, or store the sample at –20°C.

Appendix 3: cDNA synthesis

Prior to the synthesis of cDNA, the samples were diluted with RNAse free water to reach the same concentration for all samples. The sample with the lowest concentration was used as the reference. Hence, a concentration of 29,1 including 300ng, with the volume of 10 µL was used for the cDNA synthesis.

CLEAN ROOM	
Prepare DNase treatment Mix for each RNA sample	
	V(µL)
10x xRxn Buffer	1,2
Nuclease free water	12-$V_{(RNA)}$-1,2-1,2
DNase 1 Invitrogen AMPD1	1,2
Total	12-$V_{(RNA)}$

TEMPLATE ROOM	
DNase treatment mix for each RNA RT+ sample	
	V (µl)
RNA	*
DNase treatment Mix	12-$V_{(RNA)}$
Total	12
Mix gentle by pipetting up and down, incubate at room temperature for 15 minutes.	
DNase stop (EDTA)	1,2
Total RNA treated	13,2
Incubate at 70 °C for 10 minutes, chill on ice for about 5 minutes, spine down	

CLEAN ROOM	
Prepare cDNA Master Mix 1	
Number of reactions	1
Primer mix 2uM** (µl)	1,2
dNTPs mix 10 uM (µl)	1,2
Total (µl)	2,4
Primer Mix**: Even V of each (rev SGN, rev GAPDH, rev HPRT, revEEF2, rev RPS5 and Oligo DT)	

TEMPLATE ROOM	
cDNA Master Mix 1 for each RNA	
RNA treated (µl)	13,2
Master Mix 1 (µl)	2,4

Total (µl)	15,6
Anneal at 59°C for 5 minutes, put on ice >1 minute.	

CLEAN ROOM		
Prepare cDNA Master Mix 2		
	RT+	RT-
Number of reactions	1	1
5x First-Strand Buffer (µl)	4	0,8
0,1M DTT (µl)	1	0,2
Superscript 4 RT (200U/ul) (µl)	1	0
Nuclease free water (µl)	1	0,4
Total (µl)	7	1,4

TEMPLATE ROOM		
cDNA Master Mix 2 for each reaction		
	RT+	RT-
Master Mix 2 (µl)	7	1,4
Template RNA treated (µl)	13	2,6
Total (µl)	20	4
Incubate at 25 °C for 15 minutes		
Incubate at 55 °C for 60 minutes		
Inactive reaction at 70 °C for 15 minutes		

Appendix 4: qPCR Master Mix and qPCR protocol

Samples: 1:5 dilution with RNAse free water
Vessel: Bio-Rad white plate
Seal: Bio-Rad optical seal
Primerstock, uM: 10
Primerconc (final uM): 0,4
Replicates: 2

Prepare the master mixes according to the table for the given amount of reactions and for the specific gene:

CLEAN ROOM	
Preparation qPCR mix	
Per number of reactions	1
	V(µL)
SYBRGreen master mix	12,5
Primer forward	1
Primer reverse	1
Nuclease free water	8,5
Total µl mastermix per well	**23**

Template room:

- Add µl mastermix per well: 23
- Add µl sample per well: 2

Program

1. 95 °C for 5 min
2. 95 °C for 15 sec
3. 58 °C for 30 sec
4. 72 °C for 30 sec, collect SYBR green
5. Go to step 2, 39 times
6. Melt curve: 60 °C, 0,5 °C steps to 95 °C, 10 sec dwell time