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ESTABLISHMENT OF A PET TUMOR BIOBANK

ISTITUZIONE DI UNA BIOBANCA DI TUMORI DI ANIMALI DA COMPAGNIA

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ABSTRACT

Today in Italy there are 10 known veterinary biobanks which collect tumor tissue samples from pet animals, in order to have an archive available for research purposes. This work aimed at evaluating if a small Veterinary Teaching Hospital could be able to bring together a significative number of tissue samples able to sustain biomolecular research and/or join with partner Institutions to reach a common goal, and the challenges behind the establishing of a canine and feline tumor biobank.
Sample collection began in June 2022 and, until August 15th 2023, 65 different tumor samples from 5 cats and 59 dogs, out of 124 planned oncological surgeries were collected. The tumor types collected, in decreasing order of sample number included mast cell tumors, soft tissue sarcomas, adenomas and carcinomas from different organs, osteosarcomas, melanomas, and some samples classified as chronic inflammation or hyperplasia on the final histopathology report. The samples were stored in Eppendorf® tubes containing RNA-later and kept at the super cool temperature of -80°Celsius, after being refrigerated overnight at 4°C. Starting from patient 47, the collection also included the storage of blood serum withdrawn the same day of the surgery. Sampling was possible in about half of the total programmed oncologic surgeries performed, for different reasons, concerning both tumor size and logistical problems related with staff availability. So far, we can affirm that, at least for some types of tumors, such as mast cell tumors, soft tissue sarcomas and some carcinomas, the number of samples yearly collected may suffice to be used for starting a research program, although an implementation of cases would be beneficial.

RIASSUNTO

Attualmente in Italia si conosce l'esistenza di 10 biobanche veterinarie che si occupano di raccogliere e stoccare tessuti tumorali da animali domestici, al fine di avere un archivio disponibile per fini di ricerca. Questo lavoro ha l'obiettivo di valutare se un piccolo ospedale veterinario didattico può essere in grado di raccogliere un numero di campioni tissutali sufficiente a sostenere la ricerca biomolecolare, o se questo debba necessariamente unirsi con una istituzione simile per raggiungere un obiettivo comune, e le sfide che si celano dietro l'istituzione di una biobanca di tumori di cani e gatti.

La raccolta dei campioni è iniziata a giugno 2022 e, fino al 15 agosto 2023, sono stati raccolti 65 differenti tipi di tumori da 5 gatti e 59 cani, su un totale di 124 chirurgie oncologiche programmate. I tipi di tumore prelavati, in ordine decrescente di numerosità del campione, sono stati: mastocitomi, sarcomi dei tessuti molli, adenomi e carcinomi da diversi organi, osteosarcomi, melanomi e alcuni campioni che nel referto istologico finale sono stati classificati come infiammazione cronica o iperplasia.

I campioni sono stati stoccati in provette Eppendorf® contenenti come medium l'RNALater e poi conservati a una temperatura di -80°Celsius, dove potranno restare per un tempo indefinito, dopo essere stati tenuti per una notte a 4°C. A partire dal paziente numero 47, si è iniziato a stoccare anche il siero a partire dal sangue prelevato lo stesso giorno della chirurgia.

Il campionamento è stato possibile per circa la metà delle chirurgie oncologiche programmate, per diverse ragioni che riguardavano sia la dimensione del tumore sia problemi logistici legati alla disponibilità dello staff.

Finora, possiamo affermare che, almeno per alcuni tipi di tumori come mastocitomi, sarcomi dei tessuti molli e alcuni carcinomi, il numero dei campioni raccolti potrebbe essere sufficiente per iniziare un programma di ricerca; tuttavia, è necessaria l'implementazione della biobanca per raggiungere l'autonomia di ricerca.

INTRODUCTION

Cancer can be defined as a disease of the genome, finally caused by DNA alterations that change gene's structure and their functions. Viruses, mutagenic chemicals, radiation, as well as DNA replication deficiencies are also involved in the pathogenesis of cancer. Genetic injury can involve both somatic and germ line genes. Normal tissues respond to injuries and loss of substance by proliferation, and sometimes this proliferation causes an alteration of proto-oncogenes.

When altered, these proto-oncogenes can turn into oncogenes, leading to tumor development. More than a hundred oncogenes have been identified thanks to genetic analyses of neoplasms [1]. For instance, the proto-oncogene *p53* produces a protein which has low concentration in normal cells, while in tumor-derived and transformed ones it reaches higher levels. Previous studies have shown how *p53* overexpression could even cause rodent cell immortalization. [2]. The transcription factor *p53* is, in fact, involved in several tumor-suppressing pathways such as cell cycle arrest and their death and differentiation, DNA repair and cell metabolism. Those are the reasons why *p53* function is frequently impaired in human cancers [3].

An important proto-oncogene discovered in veterinary medicine is *c-KIT*, which is involved in survival and maturation of hematopoietic stem cells, melanocytes and mast cells. A study reported alteration of the DNA region coding for this proto-oncogene in dogs with mastocytoma [4].

C-myc oncogene alterations appear to be primarily associated with neoplasms of lymphoid origin, although they may also be implicated in other cancers [5].

In dogs, cancer is one of the most common causes of death, and it affects about 4 million dogs per year [6]. The diagnosis should be made as soon as possible, beginning with collection of patient clinical history, and a thorough physical examination. Patient reporting is fundamental, since different types of tumors are more likely related to the different phenotypic and genotypic characteristics of the patient itself (e.g. species, breed, sex, age, environment). These data should be given high consideration when formulating differential diagnoses, since they could be correlated with the genesis of different tumors. For instance, intact female dogs and cats show a higher risk of developing breast cancer [7], while environmental contamination with asbestos has been shown to be the leading cause of mesothelioma development in both dogs and humans [8].

Moreover, due to the genetic involvement in the biology of cancer, it is known that there is a breed predisposition to specific cancer histotypes, therefore, this information may play an important role in the diagnostic workup of the animal.

After these first steps, there is an extended list of tools which helps in reaching the final diagnosis of a specific neoplasm and to understand tumor stage and prognosis:

- Imaging techniques →radiology, ultrasound, computed tomography (CT) scan, magnetic resonance (RM) imaging
- Laboratory analyses → blood count, biochemistry and coagulation tests, macroscopic and microscopic analysis of cavity effusions, urinalysis
- Cytologic examination of the primary tumor and of the draining lymph nodes
- Histopathology
- Molecular diagnosis [9]

Molecular diagnosis deserves a special consideration for its role in cancer research and understanding. It can provide further information about neoplasms, such as their malignancy and other prognostic factors, both in qualitative and quantitative terms, starting from the analysis of their RNA or other nucleic acids and their genes [10]. More importantly, molecular biology helps in the understanding of the mechanisms of cancer development, thus allowing to act against it at an earlier stage, and/or to reach an earlier diagnosis, compared to more conventional diagnostic methods. Through the study of the different genes, it is possible to understand protein transcription mechanisms and their role in a given pathology [11].
An important example is *Ki67* gene, which protein (pKi67) is associated with proliferative activity of intrinsic cell populations in malignant tumors, allowing it to be used as a marker of tumor aggressiveness in some tumor types [12]. Protein structure and interactions are studied by proteomics, which outputs and pattern can be evaluated as biomarkers. [13].
A *biomarker* is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [14].

Biobank definition and aims

A biobank is a ''facility for the permanent preservation and storage of biological samples and associated data, which follows standardized operating procedures and provides material for scientific and clinical use'' [15].
The complete annotation of canine genome, which was implemented in recent years, gave the opportunity to use it as a model for studying and testing new diseases and their treatments to be used also in human oncology [16]. Dogs and human cancers, in fact, share similar biological behavior and response to chemotherapy, surgery and radiation therapy. [17].
Moreover, besides testing of the toxicity and safety of new compounds in dogs, for those drugs which cannot reach clinical trials in human medicine, drug companies are also considering the veterinary medicine market as a first line. [16].

Since disease progression is more rapid in dogs than humans, this allows to study the impact of novel treatments in a shorter period of time [6].
To collect samples of tissue and other biological materials useful to support the research in veterinary and comparative oncology and molecular diagnosis, the tumors excised would need to be stored permanently, so that they can be used when needed, also years after the collection. The storage method should avoid any damage to the molecular and histological structure of tissues and, first of all, to nucleic acids and make it easy to find the required specimen anytime.
For the molecular profiling of a tumor, it is useful to collect not only solid tissues but also liquid biospecimens, since cells, proteins, lipids, and metabolites contained in whole blood, serum, or saliva can function as biomarkers.
According to the IARC (International Agency for Research on Cancer), biobanks put together three fundamental pillars of medical research: molecular and genetic epidemiology, molecular pathology and pharmacogenomics/pharmacoproteomics [18].

Organization of a Biobank

When creating a biobank, according to the IARC, there are some key features that need to be considered, such as: type, number and size of biospecimens; storage containers and storing temperatures and biospecimens associated data. The tissues which will become part of the biobank can be obtained from open surgery, endoscopy or ultrasound-guided biopsies [18]. The clinical units, such as pathology and surgery, need to communicate efficiently to support biorepository operations, like patient enrolment, biospecimen and patient's data collection, final histopathology diagnosis, and finally processing and storage of the samples [19].

Biospecimens identification and data storage

An information technology system is strictly required and provides clinical information about solid tissues and liquid biomaterials [20]. Donor data and samples are uniquely linked by the code given to the sample [19]. The database provides clinical and pathological information of the biospencimens, such as: identification of the patient (name, age, sex and breed); specimen data (sampling date, histologic type, size, anatomical localization); diagnostic data (cTNM classification, lesion advancement and invasion). It is important to have data stored in two ways, both physically written on paper and on the informatic system, to avoid any data loss [21]. Moreover, every time the biospecimen is moved in or out from the biorepository, the database should be updated, to constantly have the idea of the amount of tissue of a specific sample actually available. Furthermore, it is fundamental to store samples that come from normal tissues of the same oncologic patient, to compare their DNA and to identify the cancer specific variants and their phenotypes. A lot of human sequencing centers use this method for

more accurate calling of somatic mutations using nucleic acids. Recently, the procedure of mutation profiling has begun to be integrated in veterinary research [20].

Storage

Once the mass has been removed from the host, samples must be sterilely placed in the vial as soon as possible, since the ischemia process affects the values of biomarkers detected and the integrity of RNA. *Cold ischemia time* is ''the time spent at room temperature by the tissue sample from its excision from the host to the fixation in the RNAlater solution'', and this time should not exceed 3 hours. [22].

For instance, regarding to renal carcinoma, it has been shown that there is a significant RNA degradation within 240 minutes after resection, when keeping the tissue at 37°C. In this condition, ischemia and storage method may cause the altered expression of more than 4000 genes; and the longer this time, the greater the number of genes that are altered. [23].

Warm ischemia time is, instead, 'the time between blood vessel ligation and surgical excision of the tumor". The latter depends on the surgical procedure, and this may cause delays in delivering samples to the biobank, potentially causing stress-induced biochemical changes. This time lapse should be marked on the sample data sheet and interpretation of molecular profiling should be done with great caution. [22].

Solid tissue specimens can be preserved in 4 ways: immediately frozen with liquid nitrogen; embedded in optimal cutting temperature medium and frozen on dry ice; formalin-fixed and embedded in paraffin (FFPE) or stored in vials containing RNAlater as a medium [19]. The various issues concerning each of the above preservation methods have been widely discussed in the literature:

- Liquid nitrogen provides a very stable ultra-low temperature environment but, especially during tissue processing (more than during storage), there is a potential contamination risk involving environmental and water-borne bacteria and fungi. Moreover, liquid nitrogen isothermal freezers require high running costs [24].

-Dry ice freezing may provide chemical and physical damage to cell membranes [25], moreover, it is not readily available in all Institutions

- FFPE is useful when sample quantities are limited, and this method is also suitable for several specialized techniques such as fluorescence *in situ* hybridization (FISH) and immunohistochemistry [26]. It is always available, since each sample is submitted to histopathology diagnosis, but the formalin fixation may alter nucleic acids and proteins, therefore these samples may not be suitable for all kind of analysis.

- RNAlater rapidly permeates tissues and stabilizes and protects cellular RNA and it is compatible with expression and gene-profiling data, but on the other hand, when RNAlater is used, trypsin treatments cannot be carried out, since enzyme activity is inhibited and there is more mechanical degradation during cell destruction. Moreover,

RNAlater is unusable for carrying out comparative proteomics analysis. [19]. Anyway, it is one of the best methods to preserve nucleic acids at the moment.

Limits of the Biobank

Usually, each Institution owns a biobank composed of the samples collected from the same and/or affiliated Institutions. It would be desirable that the biological material would be shared between centers, but this is rarely the case. Samples quality is very important, but there are various uncontrollable preanalytical variables that could compromise the molecular composition of tissues and compromise the analysis; these variables, which can involve both patient and storage methods, need to be carefully registered.

For example, regarding to patients, it is important to write correctly their history, particularly if they underwent chemotherapy treatment before surgery and any concomitant diseases. Regarding to storage methods, we have to consider *warm ischemia time* and storing time and temperatures. [22].

Collecting high-quality specimens is not enough, it is fundamental to reach a certain number of samples too. This latter event depends on the number of tumors seen per year by the Institution, and on their size, since a certain amount of tumor is needed for the histopathology diagnosis, thus limiting the number of small tumors available for the bank. [19].

Organizing and maintaining a biobank is therefore rather complex and time consuming, since it requires dedicated personnel in charge of adequately collect and store the samples, of filling in and update the database, and to stimulate the clinicians to collect biosamples in a proper way. It is clear at this point that there are still limitations for research progression through biobanks, and it is still a significant challenge to build a virtual biobank network among different cancer centers, trying to keep different stakeholders satisfied with their interests [27].

MATERIALS AND METHODS

Our biobank biospecimens were picked up from oncologic patients who underwent planned surgery for the removal of neoplastic lesions during the clinical activity at the Veterinary Teaching Hospital of the University of Parma.

Inclusion criteria

To be included in the biobank, sufficient size of the neoplastic masses at the time of the excision is necessary to both do not disrupt the specimen to be sent for histopathology examination and collect enough tissue to perform molecular analysis on the stored tissue; therefore, it was decided to include tumors of at least 1.5 cm in diameter.

Exclusion criteria

The main reasons for excluding patients from the sampling process are the following:

- Insufficient size of the neoplastic mass
- contamination of the tissue before collection

Sample identification and data log

Before starting to collect samples, we had to figure out a way to identify them in the long term and to set a database to collect samples information.

We created an Excel worktable (Fig. 1) where we could record patients' and biospecimens' data. The first worksheet of our Excel database is nominated ''Codes and patients' data'' in which there is a table enumerated with progressive numbers, containing donor's information (owner's name and number, animal's name, age, sex and breed) and tumor mass features, such as anatomical site and size, both at first medical examination and right before surgery, organ of origin.

For each sample at least 2 vials (Eppendorf® tubes) are included, one with the tumor and one with normal tissue (usually skin collected as far as possible from the tumor). When the tumor was large enough, more than one vial was stored and, when possible, also the sentinel lymph node was harvested.

Each vial sample (tumor and skin) has a progressive identification number, which is the same for every vial sample of the same donor, and it is written with a cryogenic permanent mark and on specific cryogenic labels attached to the vial, so that it cannot be lost after freezing.

In order to recognize the tumor sample from the healthy tissue of the same donor, we ordered round labels in two colors: red for the tumor and green for the healthy tissue (Fig.6). We apply the round labels on the lid of the tube in such a way as to immediately identify the type of tissue and the patient's code, without necessarily having to pick up

the tube from the biorepository once samples are frozen. We also apply another label reporting the patient code in the lateral part of the tubes and added an additional label with patient's name upon each Eppendorf® tube in order to better identify the sample, reducing the possibility to lose the reference: at first using a piece of tape and handwriting pet and owner's name, then printing pre-filled labels in which we can find owner's name and surname, pet's name and breed.

The excel cells reporting patient's codes are colored red, as the labels we use for the identification of neoplastic tissue (Fig.1), while the cells in which we write from where skin was harvested are highlighted in green as the labels we use for this biospecimen (Fig. 4).
In the first excel cell the patient's code and the number of tumor vials collected for each tumor are reported (i.e. 1x, 2x, 3x). If multiple organs (e.g., primary tumor and sentinel lymph node) are collected from the same animal, this is also clearly reported in the data sheet.

CODES	Animal's name and species	Owner	Breed	Cell phone	Age	sex	Tumor's localization	Tumor size at first examination	Citology exam report
1 (2x)	Piuma, dog	Camparini Monica	golden retriever	3204370625	15	F	left thoracic limb	3-4cm	issue sarcoma vs histiocytic
2 (2x)	Romeo, dog	Barilli Sabrina	Border collie	3333015188	12	M	left anal sac	4x6cm	AGASACA
3 (1x)	Verde, dog	Tavolaro Marta Lorenza	Golden retriever	33674930446	7	F	left pelvic limb (thigh)	1,5cm	mast cell tumor
4 (2x)	Ruby, dog	Crotti Mariella	mixed breed	3357546216	10	F	left 5 breast	5x4,5 cm	/
5 (2x)	Tor, dog	Grondelli Luciano	Drahtar	3933317819	8	M	left proximal humerus	/	/
6 (2x)	Bibi, dog	Craviari Afro	mixed breed	3356786833	13	MC	scrotal region	5cm	mast cell tumor
7 (2x)	Kida, dog	Bertorelli Valentina	mixed breed	3273281999	5	FS	left pectoral region	4x7cm	thelial vs mammary neoplas
8 (2x)	Cindy, dog	Lazzari Francesco	mixed breed	3287810116	13	F	middle portion of the left ra	1x3cm	soft tissue sarcoma grade II
9 (2x)	Balu, dog	Bertuletti Silvia	mixed breed	3934358011	8	MC	caudal region of the left thi	1,5cm	mast cell tumor
10 (3x)	Linda, dog	Donzella Annarita	Cocker Spaniel	3295368460	9	S	soft tissues left elbow later	3,48x2,40x7,32cm	soft tissue sarcoma
11 (2x)	Mishon, dog	Ena Andrea	Rottweiler	3338472813	6	S	5 finger left forelimb	5x8cm	/
12 (2x)	Zoe, dog	Montanari Carlo Alberto	mixed breed	3355731099	8	F	left perivulvar region	2x2,5cm	mast cell tumor
13 (3x)	Sally, dog	Bornati Alessandro	Cocker Spaniel	3391913236	14	FS	right mandibular arch	3x5cm	oral melanoma relapse
14 (2x)	Black, dog	Ferrari Enrico	mixed breed	3473365326	16	M	left mandibular arch	1,3x1,4x2,7cm (TAC 18/08/22)	oral melanoma
15 (2x)	Maggie, dog	Ziveri Corrado	Balkan bloodhound	3383154155	11	F	right knee	2,1x2,3cm	mast cell tumor high grade
16 (2x)	Fanny, dog	Agostini Valentina	mixed breed	3287293708	13	F	left prescapular region	6x5cm	mast cell tumor
17 (2x)	Emilia, dog	Savi Fabrizio	Italian spinon	3774887730	6	F	right 5 breast	12x8cm	/
18 (2x)	Max, dog	Pograri Fabiana	mixed breed	3493708263	15	M	left inguinal region	2x2,5cm	AGASACA relapse
19 (2x)	Assal, dog	Flisi Stefano	saluki	3356097100	8	F	thyroid	4,5x4,2cm	thyroid carcinoma
20 (2x)	Astor, dog	Sandri Marco	dobermann	39232114083	8	MC	right ulna diaphysis	/	osteosarcoma

Fig. 1. Patient and tumor data reported in the Excel worktable

In the same datasheet we also record preoperative cytology and/or histology diagnosis, if they have been performed, the surgical techniques used, the date of the surgery, -80°C storage date, classification, and definitive histopathological diagnosis.
To make everything more accurate, there is also an individual worksheet (Fig. 2) for each patient in which we record the clinical history and all the exams performed before and after surgery, such as blood tests, x-rays, CT scan and ultrasound reports.

Date	Anamnesis	Clinic examination	Diagnosis	Therapy
01/06/22	Dog manifests difficulty breathing when agitated, but exploration of the oral cavity is difficult due to the animal's temperament. Left shoulder region, proximal humerus with area of bony rarefaction. Presence of a ranula, already treated several times, to be evaluated sialoadenectomy constestual to amputation.	Hypotonic left forelimb muscle masses. Left popliteal lymph node increased in size.	HISTOLOGY of the mass in left prossimal homerus--> productive osteoblastic osteosarcoma.	Analgesic therapy with oral gabapentin, tramadol, and meloxicam.
		CBC examination in the normal range except for a mild thrombocytosis Blood biochemical test in the normal range		
08/06/22		CT scan: metaphyseal lesion humerus sn to be defined. Regional lymphadenopathy. Hepatization from sub pleural aspect of caudal lobe sn (interstitiopathy, atelectasis, outcome of pneumonia, neoplastic infiltrate) with perilesional atelectasis. Spondyloarthrosis, protrusions, lumbosacral stenosis. prostatic hyperplasia/reactivity.		continue analgesic and anti-inflammatory therapy and consider amputation of the limb if confirmed diagnosis of primary bone neoplasm.
27-lug		JRGERY: left limb amputation including scapula, sialoadenectomy for pharyngeal sialocel		
28-giu	blood biochemical test in the normal range			
29-giu	CBC examination: hct 29%, Hb 10gr/dl, mild leucocitosis, mild neutrofily			
	Owner report that Thor is doing well	CHEMOTHERAPY: 1stcarboplatin 300mg/mq (23ml) + maropitant 1mg/kg		
11-lug		Clinical examination: the wound is in an excellent state of healing, metal staples are removed		
20-lug		CBC examination: mild thrombocytosis		
		CHEMOTHERAPY: 2nd Carboplatin 300mg/mq (23ml) + maropitant 1mg/kg		

Fig. 2: An example of a patient's clinical history and post-surgery follow-up

These personal patient's pages can be automatically opened by clicking on the patient's own name in the initial table (column "name and species"), thanks to the hyperlink previously created on the patient's name cells.

Other reported data include the *warm ischemia time*, which is defined as the time a surgical sample is still within the body, but with a compromised blood supply [19] due to the surgical intervention. This corresponds to the minutes from the closure of the arteries to the storage of the tumor into Eppendorf® test tubes and it was recorded because it changes from one surgery to another. *Cold ischemia time* is 'the time spent by the tissue at room temperature from its excision from the animal to the actual refrigeration'' and needs to be kept to less than 3 hours, according to recent studies [22]. This time has not been recorded, since we always store the samples in the Eppendorf® test tube with RNA later within a maximum of 10 minutes after the removal of the tumor.

In the last Excel worksheet, all patients are grouped by the type of tumor they present.

CODES	Biopsy report	Pre-surgery tumor size	Chemotherapy	Surgery	Surgery's date (dd/mm/yyyy)	Storage date (dd/mm/yyyy)
1 (2x)	same as citology	6x7cm	no	Sts excision and lynfhadenectomy	13/06/22	13/06/22
2 (2x)	/	5cm	no	s excision and sacral lymphadenect	13/06/22	13/06/22
3 (1x)	/	1 cm	no	l lymphadenectomy (left popliteal a	16/06/22	16/06/22
4 (2x)	/	1,5x1x1 cm	no	lest mastectomy, Left inguinal and	20/06/22	20/06/22
5 (2x)	osteosarcoma	2x3,5cm	yes (post-op)	left forelimb amputation	27/06/22	27/06/22
6 (2x)	/	5cm	no	Mass excision at scrotal level, lymp	04-lug	04/07/22
7 (2x)	/	4x7cm	yes (post-op)	oral level + ipsilateral axillary lymph	04-lug	04/07/22
8 (2x)	/	5,1X4,7cm	yes (post-op)	left flank mass excision	07/07/22	08/07/22
9 (2x)	/	2x2cm	no	Mast cell tumor excision ipsilateral	18/07/22	19/07/22
10 (3x)	/	3,48x2,40x7,32cm	no	Left thoracic limb amputation + lef	21/07/22	22/07/22
11 (2x)	Well-differentiated squamo	5x5x2cm	no	Left thoracic limb amputation + lef	28-lug	29-lug
12 (2x)	/	1,5cm	yes (post-op)	perivulvar mast cell tumor removal	08-ago	09-ago
13 (3x)	/	4 cm	no	Right mandibolectomy and submar	08/08/22	09/08/22
14 (2x)	/	3x2,5cm	yes (post-op)	Left andibolectomy and lymphaden	22/08/22	23/08/22
15 (2x)	oma according to patnaik an	2x1,5 cm	yes (post-op)	Mast cell excision	12-set	13/09/22
16 (2x)	/	7x5cm	yes (post-op)	Mast cell excision	27/10/22	28/10/22
17 (2x)	/	same as first examination	no	right mastectomy	19/09/22	20/09/22
18 (2x)	/	2,5x2,5cm	yes (post-op)	Metatasectomy 2cm skin nodule at	19/09/22	20/09/22
19 (2x)	/	6x5cm	no	thyroidectomy	10/10/22	11/10/22
20 (2x)	/	8cm	no	Left thoracic limb amputation + lef	13/10/22	14/10/22

Fig. 3: Other important data recorded.

CODES	Histopathologic report	Healthy skin localiza	warm ischemia time	SERUM	Time until centrifugatio	age until centrif	Death
1 (2x)	Soft tissue sarcoma morphologically compatible with moderately differentiated perivascular	abdomen	<30mins	NO	/	/	NO
2 (2x)	Bilateral apocrine gland tubular carcinoma / testicular neoformation diffuse sertolioma	tail base	<30mins	NO	/	/	NO
3 (1x)	2nd grade mastocytoma (Patnaik), low grade (Kiupel)	left thigh	<30mins	NO	/	/	NO
4 (2x)	subcutaneous cavernous angioma	abdomen	<1h	NO	/	/	NO
5 (2x)	productive osteoblastic osteosarcoma	left elbow	2h	NO	/	/	NO
6 (2x)	Grade I soft tissue sarcoma morphologically compatible with perivascular sarcoma	left thigh (medial re	<30mins	NO	/	/	NO
7 (2x)	Undifferentiated sarcomatous form, poorly differentiated muscle origin	left axillary region	<30mins	NO	/	/	NO
8 (2x)	Grade I soft tissue sarcoma morphologically compatible with perivascular sarcoma	thorax	<30mins	NO	/	/	NO
9 (2x)	HN2 subcutaneous mast cell tumor	left caudal thigh	<30mins	NO	/	/	NO
10 (3x)	Grade II soft tissue sarcoma morphologically compatible with perivascular sarcoma	left forelimb caudal	1h 30 mins	NO	/	/	NO
11 (2x)	well-differentiated squamous cell carcinoma	left shoulder	1h30mins	NO	/	/	NO
12 (2x)	Grade 2 cutaneous mast cell tumor according to the Patnaik classification and low-grade acc	left thigh	<30mins	NO	/	/	NO
13 (3x)	Oral melanoma immunohistochemistry CSPG4 mild positivity, more than 75% of cells, score	right cheek	<1h	NO	/	/	NO
14 (2x)	Oral melanoma immunohistochemistry CSPG4 mild positivity, more than 75% of cells, score	left cheek	<1h	NO	/	/	NO
15 (2x)	Cutaneous mast cell tumor II patnaik, low grade kiupel, right inguinal lymph node hn2	right pelvic limb	<30mins	NO	/	/	NO
16 (2x)	Patnaik grade 3 cutaneous mast cell tumor, and Kiupel high-grade cutaneous	neck (ventral region)	<30 mins	NO	/	/	NO
17 (2x)	adenosquamous carcinoma grade II, metastatic lymnph node	1 right breast	<1h	NO	/	/	NO
18 (2x)	carcinoma of the hepatoid glands	inguinal incision mar	>30 mins	NO	/	/	NO
19 (2x)	Follicular/solid thyroid carcinoma with intravascular emboli	neck (ventral region)	<30 mins	NO	/	/	NO
20 (2x)	Mixed histotype (fibroblastic/chondroblastic) grade III osteosarcoma with micrometastasis a	right shoulder	1 h 30 mins	NO	/	/	NO

Fig. 4: Histologic reports, healthy tissue region of sampling and warm ischemia time, data column with serum data added afterwards.

The same basic information reported in the excel file are also kept in a hardcopy file, which is manually updated as soon as samples are collected, and it is collocated nearby the operating room. This paper sheet is used as the first step to relate the donor code to its information and to speed up the collection process (Figure 5). When serum collection started, we added a column indicating the collection (yes/no) of the serum sample.

CODE (+ number of red tubes)	Animal name and species (dog / cat)	Owner (name and surname)	Surgery date	Biopsy / cytology	Tumor localization	Healthy skin localization	SERUM	Warm ischemia time <30 mins / <1h <1h30 mins / <2h <2h30mins / <3h
53 (3x)	Tina (dog)	Luigi Rocca	22/05/23	AGASACA	Right anal sac	Right perineal region	YES	<30 mins
54 (2x)	Roger (dog)	Carina Zaccaro	29/05/23	/	Spleen	Abdomen	YES	<30 mins
55 (1x)	Emma (dog)	Emanuele Pettenati	05/06/23	Mast cell tumor	Left neck mast cell tumor (small)	Right neck	YES	<30 mins
56 (2x)	" "	" "	" "	" "	1x Right neck mast cell tumor (big) 1x mandibular lymph node (56L)	" "	" "	<30 mins
57 (1x)	Tommy (dog)	Alberto Tagliavini	08/06/23	/	Right elbow	Right elbow	YES	<30 mins
58 (2x)	Kira (dog)	Danilo Saccani	15/06/23	/	Spleen	abdomen	YES	<1h
59 (2x)	Gunny (dog)	Cristin Bertasi	03/07/23	Osteosarcoma	Right thoracic limb	Right shoulder	YES	<3h
60 (2x)	Penelope (dog)	Colombi Daniele	10/07/23	Squamous cell carcinoma	Tongue	Skin not drawn	YES	<3h

Fig 5. Part of the basic information collected at the time of collection. This database is available in a hardcopy form located near the surgical room.

Tissue samples collection

Samples are stored in 2 mL Eppendorf® tubes prefilled with 1.5 mL of RNAlater. RNAlater is an aqueous solution used to preserve RNA in fresh tissue and cell samples for clinical and transcriptomic studies. The compounds contained are quaternary ammonium sulphates and cesium sulphate, which stabilizes RNA, DNA and protein content by denaturation of RNases, DNases, and proteases [28].
We used the Invitrogen RNAlater Stabilization Solution purchased by ThermoFisher Scientific®. It rapidly permeates tissues and stabilizes and protects cellular RNA. Using RNAlater there is no need to immediately process tissue samples or to freeze them in liquid nitrogen. Tissue pieces can be harvested and submerged in RNAlater solution for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation.
Moreover, RNAlater brings to immediate RNase inactivation avoiding RNA degradation and its use helps reduce the need to freeze and to grind. It is convenient for field collection, allows the storage of different types of tissues, and it is compatible with gene-profiling studies.
Before freezing a -80°C, each sample is kept overnight at 4°C, which helps to maintain the stability of the tissue [20] because the medium can better penetrate within it, avoiding crystals formation.

Since the storage temperature is -80°C, we use specific cryogenic labels purchased from GA international Labtag®, a website in which there was an enormous choice of labels.

Figure 6. Round red and green labels (Labtag.com website)

From Labtag website we also bought a black cryogenic permanent marker (Fig. 7) which we use to write down donor's code both on the tube cap and on the side.

Figure 7. Alcohol resistant and cryogenic permanent black marker (Labtag.com website)

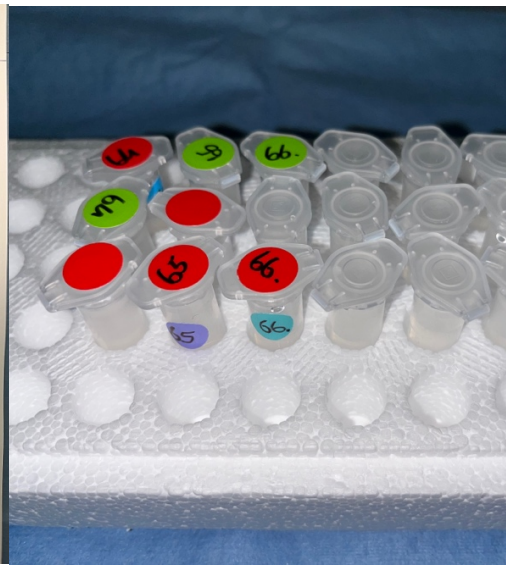

Figure 8. Labels for the preparation of the test tubes (left)
Figure 9. Stage of preparation of the test tubes before surgery (right)

Tubes are periodically filled with RNA-later and prepared so that the operator of the day can easily pick them up (Fig. 9). The code number is written in only one red tube and in the green one. Other red tubes are left unlabeled.

Whenever there is a programmed oncologic surgery, the Eppendorf® test tubes are collocated next to surgery table, waiting for the surgeon to excise the neoplastic mass.
It is fundamental that the operator responsible for taking care of the sample collection during surgery reminds surgeons to give him/her in a sterile way (Fig. 10) the tissue pieces and then the piece of skin, before finishing the skin suture.

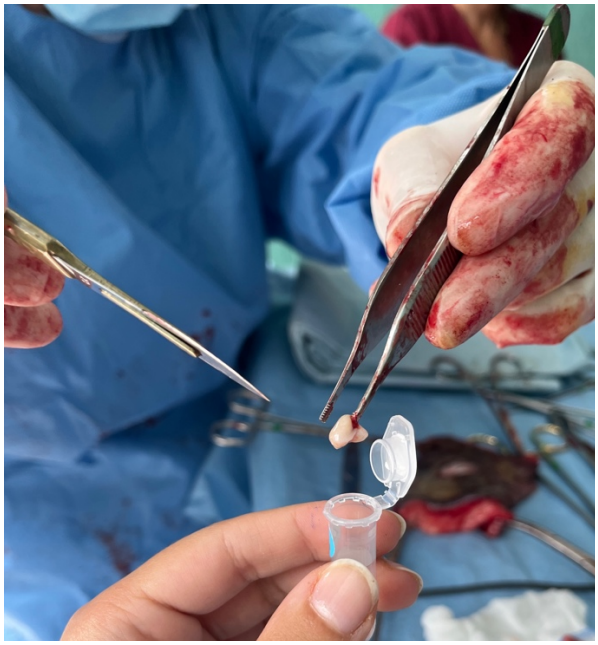

Fig. 10 Sterile placement of samples in the tubes

Samples of 0.5 cm are collected for each tumor; when the mass is large, 2 to 3 vials are filled. The tissue should be collected far from tumor margins, it should not include necrotic tissue, blood coats and irrelevant surrounding tissue; moreover, the integrity of the specimen should not be disrupted during collection, in order to allow for the histopathology evaluation, including excision margin evaluation. [19].
Once picked up, the tumoral pieces are stored in the surgery block refrigerator waiting for the surgeon to take the skin sample, since maintenance of specimens at 4°C after removal at surgery and during transportation to the pathology laboratory is somewhat beneficial in stabilizing changes in some biomolecules. [22]. For the skin we pick up a piece of about 0,5 cm and use a single Eppendorf® tube for the storage.
Once all the vials of the same animal are ready, they are put overnight in a refrigerator, letting the RNAlater to better penetrate the tissue and to prevent crystals formation, as recommended by the RNA later productors in its datasheet. The day after surgery, the samples are moved from the refrigerator and frozen at a temperature of -80°C.

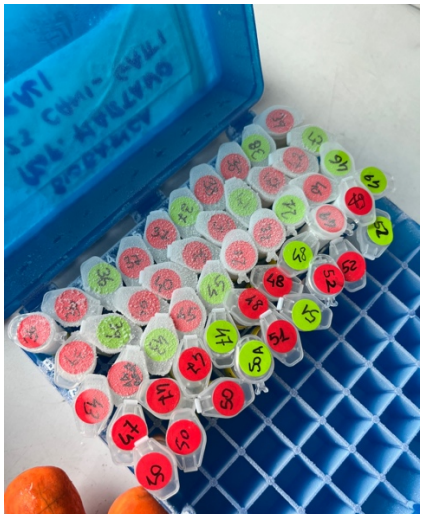

Fig. 11. Eppendorf® vials in the box for storage at -80°C.

Serum collection

At some point of our collection, we thought it might be useful for the purposes of the research, to collect patient's serum. The first serum sample was taken from patient 47; for this collection, owner must sign a written consent.

Whenever possible, the blood sample is the same withdrawn for preoperative routine bloodwork; in cases where the blood staging is performed by the referring veterinarian, the blood is collected before going to surgery, while the patient is already under general anesthesia. Usually, and according to the size of the animal, 0.5 to 1.5 ml of serum are collected.

The whole blood sample is stored in the refrigerator until the spinning time for serum collection, and the donor's code, the time we put the tube in and take it out from the fridge and the storage temperature are written on a hardcopy of the datasheet. This way we can know the length of time from blood sampling to centrifugation and we can add the information in the Excel database (Fig. 12)

CODES	Histopathologic report	Healthy skin localizat	warm ischemia time	SERUM	Time until centrifugation	rage until centrifi	Death
46 (1x)	Intestinal transmural adenocarcinoma, hyperplastic lymphadenopathy	abdomen	<1h	NO	/	/	SI
47 (3x)	Soft tissue sarcoma morphologically compatible with perivascular sarcoma (PWT)	right thigh	<30 mins	YES	1h	room temperat	NO
48 (2x)	Probable malignant myoepithelioma -> breast neoplasm with abundant mucinous/mixoid cc	back	<1h	YES	30'	room temperat	NO
49 (2x)	Grade 2 cutaneous mast cell tumor (Patnaik), and low-grade (Kiupel), HN1 and HN0 poplitea	left thigh	<30 mins	YES	30'	room temperat	NO
50 (3x)	Chronic multifocal-coalescent granulomatous enteritis and peritonitis with lymphangectasia	abdomen	<1h	YES	4h	refrigerator	NO
51 (1x) [27]	Well-differentiated thyroid carcinoma (arising in hyperplasia of follicular cells and C cells	finger	<30 mins	YES	2h	refrigerator	NO
52 (2x)	Soft tissue sarcoma, compatible with fibromyxosarcoma	back	<30 mins	NO	/	/	NO
53 (3x)	Solid adenocarcinoma of the apocrine glands of the anal sacs	left perineal region	<30 mins	YES	1h30'	refrigerator	NO
54 2x)	Splenic lymphoid and hematopoietic hyperplasia associated with multifocal hemorrhage-mu	abdomen	<1h	YES	1h	refrigerator	NO
55 (1x)	Grade 2 cutaneous mast cell tumor according to the Patnaik classification and low-grade acc	neck ventral region	<30 mins	YES	1h	refrigerator	NO
56 (2x) different	Severe chronic pyogranulomatous nodular dermatitis locally extensive	" "	<30mins	YES	30'	refrigerator	NO
57 (2x)	II grade soft tissue sarcoma	right elbow	<30 mins	YES	1h	refrigerator	NO
58 (2x)	Multiple nodules of splenic hyperplasia with associated hemorrhages, multifocal extramedu	abdomen	<30 mins	YES	1h	refrigerator	NO
59 (2x)	Poorly differentiated osteosarcoma, lymph nodes morphologically negative for metastasis.	right shoulder	1h 30mins	YES	2h	refrigerator	NO
60 (2x)	squamous cell carcinoma multiple nodules	skin not drawn	<3h	YES	2h	refrigerator	NO
61 (2x)	II grade soft tissue sarcoma	right leg	<30mins	YES	10'	room temperat	NO
62 (3x) different	62 a cutaneous mast cell tumor grade 2 Patnaik, low grade Kiupel/ 62 b subcutaneous mast	pendulous nipple	<30mins	NO	/	/	NO

Fig. 12. Excel database last columns are related to serum samples

The serum sample is stored in an Eppendorf® vial, on which we apply one of the pre-filled labels mentioned above, with the donor's code on it, and we add another label on the top of the tube with the same code.
Serum tubes are stored in a -20°C freezer indefinitely, as shown in Fig 13.

Fig. 13. Serum samples correctly identified and stored

For each biobank patient we print the final histologic report in which we write down its code and the number of red tubes used. All the hardcopies of the reports are stored in a ring binder and grouped in groups of 10. In this way we can easily go find the final diagnosis without necessarily using the computer database and viceversa.
Furthermore, in the histologic report, there is not only the diagnosis, but we can find the description of tissue structure, and this could be useful for those who will carry out future research.

RESULTS

Tissue samples collected

From the beginning of our collection on June the 13th 2022 until August 15th 2023, 65 different pathologic tissue samples and 58 healthy skin samples from 59 dogs and 5 cats has been collected. We say pathologic tissue and not ‘’tumors’’ because it happened to harvest tissue that was not neoplastic according to the final histological report but inflammation or hyperplasia. On the table below we can find the types of tissues and how many of them were collected, from the most to the least frequent.

Histological examination report	Number of samples
Mast cell tumors	12
Soft tissue sarcomas	12
Osteosarcomas	6
Mammary gland carcinomas	4
Squamous cell carcinomas	3
Sentinel lymph node for mast cell tumor	3
Canine Apocrine Gland Anal Sac Adenocarcinoma	2
Melanoma	2
Thyroid carcinoma	2
Intestinal adenocarcinoma	2
Splenic lymphoid and hematopoietic hyperplasia	2
Hepatoid gland carcinoma	1
Hepatocellular carcinoma	1
Fibroleiomyoma	1
Fibrosarcoma	1
Hemangiosarcoma	1
Transitional cell carcinoma	1
Lung carcinoma	1
Lymph node with lymphoma	1
Subcutaneous cavernous angioma	1
Malignant myoepithelioma	1
Adenomyoepithelioma	1
Thymoma (B2)	1
Gastrointestinal stromal tumor	1
Chronic multifocal-coalescent granulomatous enteritis and peritonitis	1
Chronic interstitial lymphoplasmacytic mastitis	1

Chronic pyogranulomatous nodular dermatitis	1

Patients' data

The average age of dogs is, 10.5 years old, and the median age is 10.5 Years (range 4-16 years).
The majority consists of 26 mixed breed dogs, followed by 3 Golden Retrievers, 2 Cocker Spaniels, 2 Boxers, 1 Labrador Retriever, 1 French bulldog, 1 Drathar,
1 Australian shepherd, 1 Italian foxhound, 1 Balkan bloodhound, 1 italian bloodhound,
1 Saluki, 1 Border Collie, 1 Rottweiler, 1 Beagle, 1 Pitbull, 1 Bernese Mountain dog,
1 Italian Spinon, 1 Cavalier King Charles Spaniel, 1 Dobermann, 1 Italian Poodle,
1 Podenco Ibicenco, 1 English bulldog, 1 French bulldog, 1 Lagotto.
As far as gender is concerned, intact males are 13, neutered males are 6, females are 27, spayed females are 7.
Cats are 5, with a mean age of 12.4 years and median age of 13 years (range 9-15 years). They are all domestic European cats, 3 males, 1 female, 1 spayed female.
As expected, the majority of the animals are geriatric, since the risk of developing cancers increases with age.
Thirteen patients underwent postoperative chemotherapy, 1 underwent both pre and post operative chemotherapy and 11 patients died, mostly after an euthanasia due to the worsening of clinical condition.

Biospecimens' data

Warm ischemia time was recorded for every surgery. In the table below we can see this time and for how many surgeries.

Warm ischemia time	Number of surgeries
<30 minutes	39
<1 hour	15
<1 hour and 30 minutes	3
<2 hours	1

Serum data

We started the collection of the serum from code 47 and so far serum samples are 15. All blood samples except 4 were stored at refrigeration temperature before centrifugation and the average time between picking up the samples from refrigerator and centrifugation was about 74 minutes, with a maximum of 2 hours and a minimum of 10 minutes. Ten serum samples were stored at refrigeration temperature until

centrifugation, while the others were kept for a few minutes at room temperature and centrifuged.
The elapsed time between blood centrifugation and serum freezing was not recorded because serum freezing happens within 15 minutes maximum from blood centrifugation. This information is important because tube content used to collect blood samples may affect proteins, hormones and other biomarkers and recent studies has shown that blood proteins remain stable if the centrifugation happens within 2 hours and the freezing within 2 hours after centrifugation [20].

DISCUSSION

The total number of planned oncologic surgeries from the beginning of the storage until August 15th 2023 was 124. Sampling was possible for the 47,5% of surgery consisting in tumor excision.

There were different reasons why half of all the oncologic masses excised have not become part of the collection and the most frequent one was their insufficient size, particularly when it was smaller than 1.5 cm diameter, as it happened in 2 cases of parathyroid tumors.

In fact, it is of primary importance to have the amount of tissue sufficient to be able to give a satisfactory histological diagnosis, and by using part of a small tumor for biobank storage there would be a risk of not having a complete view of the structure of the mass. In addition, for most tumors, it is critical to leave the margins intact so that the pathologist can report information to the oncologic surgeon about whether the tumor was properly removed and whether there are infiltrated margins. Sometimes the excised tumor tissue is intended in part to be used for other research projects and additional diagnostics, so we prioritized them at the expense of storage; this is to get a better identification of the tumor in question and an idea of the patient's prognosis and the best treatment to be given.

About 12 surgeries consisted in mast cell tumors excision, which dimensions were too small.

About 5 surgeries were mastectomies for mammary gland tumors that were too small for collection. Two surgeries consisted in the removal of AGASACA, and an hepatoid cell carcinoma, excluded for the small size of the tumors.

Finally, 3 surgeries consisted in lipomas removal and, in this case, collection was not planned because they are benign tumors.

Another critical point of moving forward with the collection was the staff availability: about 5 tumoral pieces were not collected because there was not available personnel to store pieces at the right temperature at the right time.

Ultimately, the main reason for not collecting the tumors was their size. This fact may create a bias, since bigger tumors may have areas of necrosis, which may be erroneously collected, thus nullifying the usefulness of the sample. Moreover, the size may be related to the aggressiveness of the tumor, again creating a selection bias in the population analysed.

Warm ischemia time is another important variable to focus on.

It was recorded because it may create a bias due to different duration of the avascular surgical phase related to the ease in removing the tumor, which may be related both to the aggressiveness and the location of the tumor itself.

Most of the oncologic surgeries performed consisted in the excision of cutaneous/subcutaneous masses, therefore *warm ischemia time* was in this cases less than 30 minutes. In the case of tumor masses whose removal involves more aggressive and complicated surgeries, such as limb amputations, mastectomies and mandibulectomies, *warm ischemia time* is longer since a longer vessel closure time is required.

As far as the serum is concerned, the fact that its collection began later than that of the samples might be a limitation for the start of certain types of studies, making it necessary to collect additional tissue and correlated serum samples. For most patients, blood centrifugation took place within the 2-hour time limit; there were two cases in which this time was exceeded, and the causes were the shortage of personnel during those surgeries and the need to proceed quickly with the surgical procedure and the animal's awakening, so the blood centrifugation was performed at the end of surgery.
Another critical point may be the anatomical part from which the skin is taken. The ideal localization is as far as possible from the pathologic tissue. But most of the time for both cosmetic and medical reasons, the skin comes from the farthest margin of the surgical wound, making possible for the pathologist to find some of tumoral infiltration in those skin samples, and likely compromising the integrity of the studies that will be conducted.
It is also important to underline the fact that data recorded in this biobank could eventually be used to start epidemiological studies regarding the onset of different types of tumors in the various breeds of domestic animals that are part of our population.

CONCLUSIONS

The main critical point in building a biobank was tumor dimensions. A reason could be the increasing awareness and attention of the owners toward their animals which brings to the early detection of these diseases.
Less important is the management of problems such as staff availability and organization and biospecimens management. There is the need for staff available and a certain level of awareness on what must be done and when to do it. Another important point that needs to be improved could be the availability of storage equipment at the direct disposal of oncology surgery staff, reducing downtime and speeding up the storage process.
Despite the problems encountered during collection, we were able to achieve a satisfactory number of samples; the most frequent tumors were mast cell tumors, soft tissue sarcomas and different types of carcinomas.

Mast cell tumor is one of the most frequent malignant cutaneous tumors in dogs and it represents around the 17.8% of cutaneous neoplasia [29].
In our case, mast cell tumors represented the 18.4% of the whole samples we stored so far and considering the number of masses excluded from storage because of their insufficient size, the percentage would certainly be much higher.
Regarding soft tissue sarcomas, according to literature, they comprise approximately 15% of all skin and subcutaneous tumors in the dog and approximately 7% of all feline skin and subcutaneous tumors [30]; they represent the 18.4% of the total masses excised in our biobank.
Mammary carcinomas are, in intact adult female dogs, the most common neoplasm, and they represent the 17% of all feline neoplasms [31]; anyway, they are of very small size in a high number of cases, therefore they are underrepresented in our biobank, in spite of being a quite common tumor treated surgically.
In fact, our population is represented by 45% of intact adult female dogs, mammary carcinoma was found in only 6% of cases.
As we can see, the database provided by the establishment of this biobank has already begun to provide us with epidemiological data, as we record patients' biographical data and their medical history and follow-up. All this information, in fact, could be used in the future to sustain scientific research concerning not only clinical oncology, but also other branches of veterinary medicine, including the study of the correlation between the occurrence of certain diseases, patient's environment and biographical data (as breed, sex and age).
Moreover, it is important to underline that storing samples at ultra-low temperatures allows their preservation for a very long time, and this allows their use for even retrospective studies concerning the molecular biology of the pathologic tissues.
In conclusion, we can say that creating and managing a biobank is challenging and requires time and dedication and it represents initially a large investment in terms of costs due to the specific facilities which requires; but although it has only recently been established it has already provided with an important base from which to start possible studies in both epidemiology and veterinary/comparative oncology.

REFERENCES

1. Meuten D. J. (2017) Tumors in Domestic Animals. Fifth edition. Raleigh, NC, USA: Wiley Blackwell, p.1-13
2. Finlay CA, Hinds PW, Levine AJ. (1989) The p53 proto-oncogene can act as a suppressor of transformation. Cell. 57(7):1083-93
3. Liebl MC, Hofmann TG (2021) The Role of p53 Signaling in Colorectal Cancer. Cancers (Basel). 13(9):2125
4. Zemke D, Yamini B, Yuzbasiyan-Gurkan V. (2001) Characterization of an undifferentiated malignancy as a mast cell tumor using mutation analysis in the proto-oncogene c-KIT. J Vet Diagn Invest.13(4):341-5
5. Alitalo K, Koskinen P, Mäkelä TP, Saksela K, Sistonen L, Winqvist R. (1987) myc oncogenes: activation and amplification. Biochim Biophys Acta. 907(1):1-32.
6. Gardner HL, Fenger JM, London CA. (2016) Dogs as a Model for Cancer. Annu Rev Anim Biosci.Epub 2016;4:199-222.
7. Canadas-Sousa A, Santos M, Leal B, Medeiros R, Dias-Pereira P. (2019) Estrogen receptors genotypes and canine mammary neoplasia. BMC Vet Res. 15(1):325.
8. Lippmann M. (1988) Asbestos exposure indices. Environ Res. Jun;46(1):86-106.
9. Couto G, Moreno N. (2013) Canine and Feline Oncology. From Theory to Practice, Saragozza – Spain, Servet, (p. 6-9)
10. Loeb KR, Loeb LA. (2000) Significance of multiple mutations in cancer. Carcinogenesis, 21(3):379-85
11. Végvári A, Welinder C, Lindberg H, Fehniger TE, Marko-Varga G. (2011) Biobank resources for future patient care: developments, principles and concepts. J Clin Bioinforma. 20111(1):24
12. Li LT, Jiang G, Chen Q, Zheng JN. (2015) Ki67 is a promising molecular target in the diagnosis of cancer (review). Mol Med Rep. 2015(3):1566-72.
13. Lee JM, Han JJ, Altwerger G, Kohn EC. (2011) Proteomics and biomarkers in clinical trials for drug development. J Proteomics ;74(12):2632-41
14. AtkinsonAJCW, DeGruttolaV, DeMetsDL, DowningGJ, HothDF, Oates JA, et al. (2001) Biomarkers and Surrogate Endpoints: Preferred Definitions and Conceptual Framework. Clin Pharmacol Ther; 69:89–95.
15. Hewitt R, Watson P. (2013) Defining biobank. Biopreserv Biobank. Oct;11(5):309-15.
16. Aresu L, Buracco P, De Maria R, Iussich S, Martano M, Morello E, Bettini G, Comazzi S, Riondato F, Marconato L. (2019) The Italian-Canine Cancer Biobank: Our 10-year challenge. Hematol Oncol. 2019 Aug;37(3):314-315.
17. Paoloni M, Khanna C. (2008) Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer. Feb;8(2):147-56.

18. Annaratone L, De Palma G, Bonizzi G, Sapino A, Botti G, Berrino E, Mannelli C, Arcella P, Di Martino S, Steffan A, Daidone MG, Canzonieri V, Parodi B, Paradiso AV, Barberis M, Marchiò C; Alleanza Contro il Cancro (ACC) Pathology and Biobanking Working Group. (2021) Basic principles of biobanking: from biological samples to precision medicine for patients. Virchows Arch. ;479(2):233-246.
19. Pelagio G, Pistillo D, Mottolese M. (2011) Minimum biobanking requirements: issues in a comprehensive cancer center biobank. Biopreserv Biobank. 9(2):141-8.
20. Talu CK, Toper MH, Şahin Y, Erdoğdu İH. (2020) Pathology and Biobanking. Turk Patoloji Derg. ;36(2):93-108.
21. Mendy M, Lawlor RT, van Kappel AL, Riegman PHJ, Betsou F, Cohen OD, Henderson MK. (2018) Biospecimens and Biobanking in Global Health. Clin Lab Med. Mar;38(1):183-207.
22. Grizzle WE, Otali D, Sexton KC, Atherton DS. (2016) Effects of Cold Ischemia on Gene Expression: A Review and Commentary. Biopreserv Biobank. Dec;14(6):548-558.
23. Liu NW, Sanford T, Srinivasan R, Liu JL, Khurana K, Aprelikova O, Valero V, Bechert C, Worrell R, Pinto PA, Yang Y, Merino M, Linehan WM, Bratslavsky G. (2013) Impact of ischemia and procurement conditions on gene expression in renal cell carcinoma. Clin Cancer Res. 1;19(1):42-9.
24. Mirabet V, Alvarez M, Solves P, Ocete D, Gimeno C. (2012) Use of liquid nitrogen during storage in a cell and tissue bank: contamination risk and effect on the detectability of potential viral contaminants. Cryobiology. 64(2):121-3
25. Til D, Amaral VL, Salvador RA, Senn A, Paula TS. (2016) The effects of storing and transporting cryopreserved semen samples on dry ice. JBRA Assist Reprod. 1;20(4):217-221.
26. Romero-Pérez L, Grünewald TGP. (2021) Tissue Preservation and FFPE Samples: Optimized Nucleic Acids Isolation in Ewing Sarcoma. Methods Mol Biol. 2226:27-38.
27. Zhang L, Wu X, Hu Y, Wang X, He Z, Xie Y, Pan K, Wang N, Dong Z, Zhang L, Ji J. (2015) Establishment of a network-based intra-hospital virtual cancer biobank. Biopreserv Biobank. ;13(1):43-8.
28. Zhu Y, Mullen AM, Rai DK, Kelly AL, Sheehan D, Cafferky J, Hamill RM. (2019) Assessment of RNAlater® as a Potential Method to Preserve Bovine Muscle Proteins Compared with Dry Ice in a Proteomic Study. Foods. ;8(2):60.
29. Oliveira MT, Campos M, Lamego L, Magalhães D, Menezes R, Oliveira R, Patanita F, Ferreira DA. (2020) Canine and Feline Cutaneous Mast Cell Tumor: A Comprehensive Review of Treatments and Outcomes. Top Companion Anim Med. Nov;41:100472.
30. Ehrhart N. Soft-tissue sarcomas in dogs: a review. J Am Anim Hosp Assoc. 2005 Jul-Aug;41(4):241-6.
31. Burrai GP, Baldassarre V, Brunetti B, Iussich S, Maniscalco L, Mariotti F, Sfacteria A, Cocumelli C, Grieco V, Millanta F, Paciello O, Papparella S, Rasotto R, Romanucci

M, Zappulli V. (2022) Canine and feline in situ mammary carcinoma: A comparative review. Vet Pathol.;59(6):894-902.