"HEART OF DOBERMANN" PROJECT

INSTITUTIONS INVOLVED IN THE RESEARCH:

1. **AIAD** – Associazione Italiana Amatori Dobermann (Dr. A. Polifrone); this institution commissions the research.

2. **ENCI** – Ente Nazionale Cinofilia Italiana; this institution finances the research.

ATTENDEES:

OVUC – Ospedale Veterinario Universitario Didattico di Perugia (Servizio di Cardiologia - Prof. F. Porciello, Dr. F. Birettoni, Dr. D. Caivano, Dr.ssa M. E. Giorgi e Servizio di Anatomia Patologica - Prof. E. Lepri)

OVIC - Osservatorio Veterinario Italiano Cardiopatie (Dr. P. Ferrari, Prof.ssa M. Longeri Dipartimento di Medicina Veterinaria Università di Milano)

Veterinarians: Dr.ssa M. C. Pisu, Dr. G. Martini, Dr.ssa V. Imbalzano

PROJECT DURATION: minimum duration is 36 months from the signing of the agreement by all Institutions involved and relevant financing.

Aims of the present study are:

1. Collection of blood samples and heart tissues during autopsy procedures for following molecular investigations from the highest number of dead Dobermanns.
2. Use of a Next Generations Sequencing technology (NGS-technology) to identify new gene mutations of Dilated Cardiomyopathy (DCM) in Dobermann.

3. Creation of an Epidemiology Database of clinical, instrumental and pathological data to certify the exemption from DCM, to include DCM-free Dobermanns in the ENCI Breeding program respecting AIAD's selection requirements.
4. Creation of a Gamete Cryopreservation Bank of Dobermanns with the highest genetic value and DNA storage for future uses and studies.
5. Determining cause of death and verifying the clinical diagnosis of DCM. Histopathological analysis will be used to evaluate morphological pattern of DCM and to apply those histological characteristics on screening Endomyocardial biopsy (EMB).

   Myocardial specimens will be stored for future biomolecular investigations.

Introduction and state of the art:

Over the last years, the interest of scientists in both human and veterinary medicine was focused on evaluation of early morpho-functional and biochemical markers of diseases. Furthermore, this interest has oriented clinicians to focus mainly on preventive medicine, as in genetic diseases, and on therapies whose purpose is to slow down the evolution of disease. To this regard, researches are focused on new diagnostic and/or new advanced technology prognostic biomarkers.

The aim of our study is to find new earlier disease biomarkers using mini-invasive screening methodologies to detect early pathological and/or functional features, to apply specific aetiological treatments and to prevent such diseases through the identification of disease carriers or more predisposed subjects.

In this context, the proposal of Prof. Francesco Porciello and Dr. Gianluca Martini is to apply a cardiological screening in breeders to improve the genetics of one the most loved and noblest dog

breeds breeded in Italy, the Dobermann, in cooperation with AIAD (Associazione Italiana Amatori Dobermann) and its President Dr. Attilo Polifrone.

Data will be used to constitute an epidemiological profile of the population. Data will be collected through traditional means like echocardiographic examinations and innovative ones like Holter recording, serum and tissue biomarkers. Moreover, the possibility to perform endomyocardial biopsy (EMB) by transthoracic echocardiography-guide with intravenous catheter will be evaluated. At present this procedure is not performed in dog cardiology.

The EMB of dogs with or without DCM will be performed in a large dog population. Using NGS technologies it will be possible to identify new genetic mutations related to the disease that might be already known or will be detected during the genomic sequencing foreseen by this project. These mutations could be identified in the blood or in the cardiac tissue and could be useful in the clinical evaluation of the single case or in the selection of the breeders.

All voluntarily enrolled participants in the study from Italian Dobermans dog breeders will be asked to be involved in two ways: evaluation of end-of-career breeding dogs and evaluation of new early-stage breeding dogs.

CLINICAL CHARACTERISTICS OF DCM

The progression of the disease (DCM) can be divided in three stages:

1. **ASYMPTOMATIC PHASE.** To date there are no clinical diagnostic tools (laboratory or instrumental analysis) able to identify asymptomatic patients. **This group might contribute to epidemiologic data through genetic tests identifying the affected subjects.** Moreover, in this asymptomatic group of animals all instrumental examinations should be used to collect **useful data for an early diagnosis**.

2. **OCCULT PHASE.** At this stage the dog owner is not able to recognize clinical signs, while the **veterinary cardiologist can diagnose the disease (DCM) by application of ECG-Holter 24H, echocardiography and cardiac biomarkers. In this phase, EMB do not have histological features characteristics of the cardiac disease, although could recognize other lesions (subendocardial fibrosis or myocarditis, etc.) that might produce symptoms similar to DCM.** About 1/3 of this group of patients encounters sudden death (malignant arrhythmias). In selected patients the therapy **could help reducing the incidence of this eventuality.**

3. **CLINICAL PHASE.** In this phase owners can notice signs and symptoms of heart failure: **a)** fatigue, wobbling, collapse; **b)** tachypnea, ascites (increasing of abdominal volume), weight loss. **The veterinary cardiologist diagnoses DCM at III stage evaluating symptoms and signs of disease and using blood markers and instrumental data (ECG and echocardiography). In this stage of disease there are histological signs in cardiac tissues (interstial and subendocardial fibrosis) evaluable by EMB** . About one-third of this population dies for sudden death (malignant arrhythmias). In the remaining two-thirds the evolution of the disease is characterized by progressive heart failure's signs. Many different drugs have to be administered in III stage patients to control symptoms, such as antiarrhythmics, diuretics, vasodilators, etc. **These kinds of drugs can't cure the disease, they just slow down the progression**.

PREVALENCE: CLINICAL SIGNS OF DCM DIFFER A LOT IN RELATION TO THE AGE OF THE POPULATION AND INCREASE IN ADULTHOOD AND OLD AGE.

GENETIC CHARACTERISTICS OF DCM.

The DCM is characterized by a strong genetic predisposition. At the beginning DCM in Dobermans was described and classified as an autosomal dominant inherited disease with incomplete penetrance (Meurs et al,2002). Moreover, some variations/mutations were described in association with DCM, although not in a complete way.

The first genetic risk factor has been localized in two different populations, one from Germany and one from Great Britain, in chromosome 5 in a region of 3 Mb (more significant variation point is CFA:g.53,941,386T.C) (Mauseberg et al 2011).

In the British population a deletion of a splice site in the canine pyruvate dehydrogenase kinase 4 (PDK4) localized in chromosome 14 was found to be the cause of DCM with a penetrance by 68% (Meurs et al., 2012). However, the same deletion searched in the German population was not associated with the disease (Owczarek-Lipska et al., 2012).

Finally, a second mutation named "DCM2" was found, but this research was not published and it is property of the University of Carolina (Meurs Lab at North Carolina State University webpage and webinar on dilated cardiomyopathy: https://cvm.ncsu.edu/genetics/doberman-pinscherdilatedcardiomyopathy/). About this mutation it is only known that DCM2 is localized in a sarcomeric gene by a 50% penetrance; penetrance can raise up to 60% if DCM2 is combined with the PDK4 mutation. Anyway, in sporadic cases DCM can show evidently in patients without these kind of mutations (Stern &Ueda, 2018).

These data show how DCM is not caused by only one single mutation and suggest that the genetic etiology could be heterogeneous and probably linked to the genetic backgroung of breeders and

their ancestors. In other words the provenance of the founders of the different genetic lines may be determining in the transmission of a different acquired genetic inheritance predisposing to the pathology.

The likely polygenic pathogenesis of DCM and the genetic stratification of the canine Dobermann population in subpopulations explain why a genomic approach could be useful in the Italian Dobermann population , using Next Generation Sequencing (NGS) technologies that allow a complete genomic sequencing, hence the analysis of gene mutations.

IMPLEMENTING PROCEDURES OF THE RESEARCH

OVIC, OVUD and AIAD are supervisors and data protector authorities during the experimental stages of the research (Items A,B,C,F that follow here below) . Moreover, they ensure that the epidemiological screening (ref.item A) will be performed through a veterinarians net validated by by OVIC and that the relevant personal data and clinical tests results will be processed in accordance with the veterinary Professionals and Clients relationship rules .

AIAD commits to broadcast all scientific results from this research in the scientific field and the Dobermann environment in order to reduce the spread of DCM in Dobermanns.

ENCI commits to support the epidemiological screening and the different stages of the research through an appropriate funding based on the costs forecast that are listed in the next pages , in order to improve the condition of the Dobermann Breed; it commits also to broadcast all the results trying to improve the conditions of other dog breeds affected by this disease.

Experts in Reproductive Physiopathology and Technologies commit to collect, evaluate and cryopreserve all the seminal fluid samples of stallions considered suitable by AIAD; they will be asked to create and supervise a CENTER FOR THE CRYOPRESERVATION OF THE SEMEN OF STUD

DOGS WITH THE MOST HIGH GENETIC VALUE, as established by the reference Enci Regulations in the process of definition at the competent offices.

UNIMI commits to develop the research on the genetic basis of the disease, in accordance with the current animal welfare legislation.

STAGES OF THE REASEARCH IMPLEMENTATION

STAGE 1: SCREENING AND GENETIC ANALYSIS

A: collection of clinical and genetic data in the OVIC Database from the first cardiological visit which has to be performed **before the reproductive age (minimum age to start screening: two years).** Breeder dogs from two years of age has to undergo a cardiological visit every year during all their reproductive life. The implementation and data processing by OVIC are required for the scientific aim. Owners will be charged 160 euros+VAT+ENPAV in agreement with AIAD-OVIC.

All the screened subject will undergo:

1. A standard echocardiographic examination. This examination will require an accurate evaluation of the left and right ventricular functions by echocardiographic indices including MAPSE (mitral annular plane systolic excursion), TAPSE (tricuspid annular plane systolic excursion), left ventricular ejection fraction and volume using Simpson's rule, left ventricular length and area, left ventricular end-systolic and end-diastolic diameters in m-mode, EPSS (E point to septal separation) length, left ventricular sphericity index, left ventricular FAC (fractional area change), RVOT-FS (right ventricular outflow tract-fractional shortening). Moreover, innovative procedures as tissue Doppler and Speckle Tracking will be used to evaluate left and right ventricular function; in particular they will be used to examine movements of mitral and tricuspid annuli, also

evaluating ventricular septum and free wall deformations. All echocardiographic measures will be related to pathologic, histologic and immunohistochemistry analysis to search for possible different alterations of the whole ventricles, of the free wall or of the only interventricular septum in patients at different stages of the disease;

2. A standard electrocardiogram

3. A 24H Holter recording

At every cardic evaluation the dog's owner will be given :

1) Echocardiographic report
2) Electrocardiogram report
3) 24H Holter recording
4) Yearly OVIC DCM exemption certificate

B: collection of EDTA blood samples (1,5ml) from all dogs during the cardiological visit and also from dogs in show, ZTP or Italians Selections Tests. This sample along with other possible tissue samples will be stored for the present and also for future researches.

Storage, validation of known mutation and research of new mutations in all collected samples will be free of charge for owners (at the research charge). For each patient one EDTA blood sample will be stored at RepA of UNIMI (see the attached A annex). This storage procedure will be free of charge for the dog's owner because samples will be considered property of UNIMI; if ENCI or AIAD will be asking for property every sample will cost 10 euros (VAT included).

C: Implementation of a complete medical examination and evaluation of the reproductive system of stud dogs; collection and macroscopic/microscopic evaluation of semen; evaluation of fertility

indexes and tests for male gametes cryopreservation. Preparation and liquid nitrogen storage of collected samples of semen from suitable dogs (see attached B annex) to constitute and to manage the **AIAD Central Semen Cryobank** .

D: Dogs' owners sharing this research program may commit to grant the autoptic analysis of the heart tissues of all dogs whose death will be **related or not to cardiological reasons** . **The autoptic analisys will be performed at the Ospedale Veterinario Universitario Didattico of Perugia (Italy) or if not possible, limited to the only heart, at any other affiliated facility . Hearts will be preserved in 10% formalin and sent in the shortest possible time to OVUC. Storage and sending methods will be agreed**. Owners will not be charged for collection, preservation, transportation and analysis of autoptic samples as they will be at the research charge . Dead dogs tissues will be analyz according to a specific protocol. In case immunohistochemistry analysis could be used and the heart hystopathologic analysis implemented to search molecules responsible for the pathogenesis and the progression of the disease. Heart tissues will be stored at -80°C for future biomolecular analysis. The histopathological investigation aims at identifying lesion patterns and possible morphological variants that can be evaluated in a second stage by endomyocardial biopsies. Portions of myocardic tissue will be stored at – 80° for possible future biomolecular reasearches.

STAGE 2: PROSPECTIVE APPLICATION OF STAGE 1 SCIENTIFIC RESULTS

E: In this second stage of the research, some selected patients, whose owners will be asking for euthanasia because of the end-stage **cardiologic** or end-stage **no cardiologic** illnesses, could undergo for a right and left endomyocardial biopsy during the anaesthesia before euthanasia (as per clause D) . Owners will not be charged. These procedures will be financed by the research (preferential price approx. 25 euros each case).

The prosecution of the experimental stage (E clause) of the research could be performed on high risk dogs to achieve an early diagnosis of DCM. This application has still to be approved by the Ministry ,therefore it remains pending. All related costs are not predictable at the moment.

- Aim of the present study is to identify related and, hopefully, causative mutations for DCM in the Italian population of Dobermanns using NGS approach. NGS methodologies can also verify the presence of known and already published mutations.

- Two groups of patients will be arranged: dogs with evident disease and dogs with asymptomatic disease. Ten dogs from each group will be selected basing on the lower degree of kinship, the most extreme phenotype, the same age and breeding context.DNA from these 20 dogs will be extracted from the blood samples of the RepA Bank of UNIMI. Two pools of DNA will be produced, one from each of the two groups. Every dog will be represented with the same quantity of genomic material. Sequencing libraries will be created and a biological barcode will be assigned to every dog to distinguish their personal genomic contribution to the variability pool. Sequencing depth will be produced with a coverage of 100x.

- All genetic variations produced will be genotyped. The final number of all variations through NGS work is still unknown, as the number of dogs that will be involved in the present study has not been determined yet . If many variations will be produced and many dogs to screen a Next Gen approach also in genotying will be useful to reduce costs.

An intellectual property agreement will be signed by all parts involved in the project to regulate the use of all scientific results in a clear way .

All parts involved in the project commit to develop the research according to the current animal welfare legislation and to request authorizations for the development of the study.