1. PHD PROJECT DESCRIPTION (4000 characters max., including the aims and work plan)

Project title: The role of DNA methylation in mitral valve disease in CKCS dogs.

1.1. Project goals

a) To investigate the CpG methylation profile associated with MMVD in CKCS.
b) To investigate the influence of epigenetic age on the development of MMVD in CKCS and compare it with telomere length.
c) Detection of the presence and level of epigenetic ageing acceleration in dogs with MMVD.
d) Examination of the cytokine profile in the study groups

1.2. Outline

Introduction

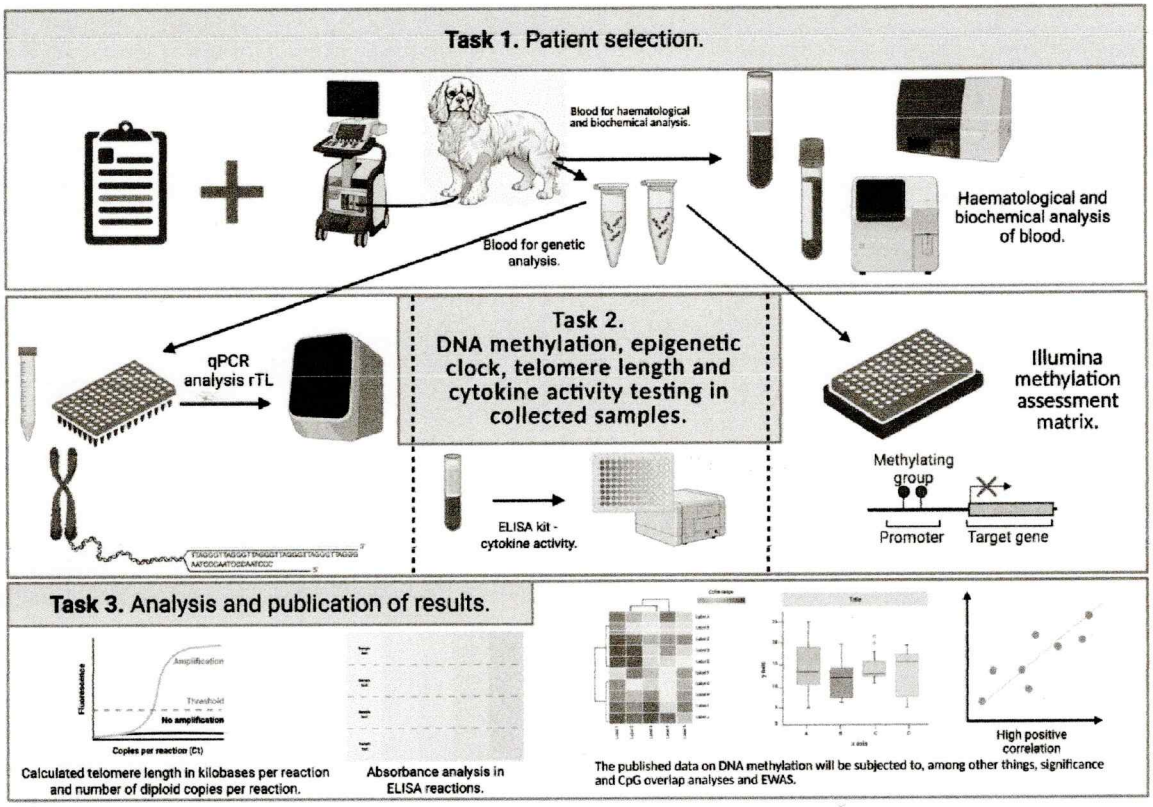
The subject of the project is DNA methylation in dogs with Myxomatous Mitral Valve Disease (MMVD). MMVD is the most common heart disease in dogs. It occurs particularly frequently in small breeds, but the Cavalier King Charles Spaniel (CKCS) stands out as a breed with an exceptionally high incidence of MMVD. The primary clinical symptom in dogs affected by MMVD is a systolic heart murmur. Genetics has been shown to play an important role in MMVD, and selective breeding may reduce the incidence of MMVD in CKCS. In particular, it is suggested that this disease is inherited as a polygenic trait. Numerous studies have been conducted to identify and elucidate the genetic and molecular mechanisms underlying MMVD. However, the methylome has never been studied before. Modifications of expression by epigenetic processes are considered particularly important. Behind methylome changes is the ability of methyltransferase enzymes (DNMT-1,2,3A,3B) to catalyse the transfer of a methyl group to cytosine or adenosine. Cytosine is methylated in so-called cytosine islands (CpG), i.e. probes, or their border regions.

By determining the methylation of CpGs in the DNA chain, it is possible to determine the epigenetic age in both humans and animals. Epigenetic age is measured using marker CpGs and is one of the measures of biological age. In many diseases, a growing discrepancy between epigenetic and chronological age has been correlated with an increased risk of cardiovascular disease. The discrepancy between epigenetic and chronological age is termed epigenetic age acceleration (EAA). EAA can be positive or negative, and positive values are associated with a higher probability of death or a poorer prognosis.

Importantly, mDNA has been shown to interact with pathways that control telomere length. Telomeres are a way of protecting DNA from damage during cell division. When the relative length of telomeres (rTL) becomes too short to allow the cell to divide safely, programmed cell death occurs. However, as telomeres shorten, p53 expression is activated and causes mitochondrial dysfunction. Energy deficiency and the accumulation of reactive oxygen species can induce the development or progression of diseases. rTL is associated with cell ageing, genetic diseases, cancer and chronic diseases such as cardiovascular pathologies. Accordingly, shorter telomeres in dogs have been associated with an increased risk of heart disease.

mDNA and the estimation of biological age in dogs is a relatively new topic that should be developed due to its cognitive, clinical, and translational nature.

Figure 1. Research design



1.3. Work plan

a) Patient selection [from 1 month to 30 months]. The first task is to select dogs from among those examined by echocardiography. In Cavalier King Charles Spaniel dogs undergoing echocardiography during a veterinary visit, blood will be collected for diagnostic testing and genetic analysis. Based on biochemical and haematological results, dogs with blood tests indicating severe disease will be removed from the sample group.

b) DNA methylation, epigenetic clock, telomere length and cytokine activity testing in the samples collected [from 6 months to 30 months]. Selected blood samples will be analysed.

   a. Telomere length: telomere determination by qPCR.

b. DNA methylation: The probes used will be matched to the dog's genome using a conservative probe package. Changes in DNA methylation will be investigated for association with age.

c. Cytokine profile analysis. Cytokines (IL-1,2,6) and TNF-α will be assessed. ELISA kits will be used for this purpose.

c) Analysis, publication and dissemination of project results [from month 12 to month 36]. This stage of the task will involve writing and publishing the results in recognised scientific journals from the JCR list with Open Access. In addition to publications, presentations at an international scientific conference are planned.

1.4. **Literature** *(max. 7 listed, as a suggestion for a PhD candidate preliminary study)*

Reimann, M. J., Cremer, S., Christiansen, L., Ibragimov, E., Gao, F., Cirera, S., ... & Karlskov-Mortensen, P. (2024). Mitral valve transcriptome analysis in thirty-four age-matched Cavalier King Charles Spaniels with or without congestive heart failure caused by myxomatous mitral valve disease. *Mammalian Genome*, *35*(1), 77-89.

Bionda, A., Cortellari, M., Bagardi, M., Frattini, S., Negro, A., Locatelli, C., ... & Crepaldi, P. (2020). A genomic study of myxomatous mitral valve disease in Cavalier King Charles Spaniels. *Animals*, *10*(10), 1895.

Horvath, S., & Raj, K. (2018). DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nature reviews genetics*, *19*(6), 371-384.

Horvath, S., Lu, A. T., Haghani, A., Zoller, J. A., Li, C. Z., Lim, A. R., ... & Ostrander, E. A. (2022). DNA methylation clocks for dogs and humans. *Proceedings of the National Academy of Sciences*, *119*(21), e2120887119.

Wang, T., Ma, J., Hogan, A. N., Fong, S., Licon, K., Tsui, B., ... & Ideker, T. (2020). Quantitative translation of dog-to-human aging by conserved remodeling of the DNA methylome. *Cell systems*, *11*(2), 176-185.

Thompson, M. J., Horvath, S., & Pellegrini, M. (2017). An epigenetic aging clock for dogs and wolves. *Aging (Albany NY)*, *9*(3), 1055.

Fick, Laura J., Gordon H. Fick, Zichen Li, Eric Cao, Bo Bao, Doug Heffelfinger, Heidi G. Parker, Elaine A. Ostrander, and Karl Riabowol. "Telomere length correlates with life span of dog breeds." *Cell reports* 2, no. 6 (2012): 1530-1536.

1.5. Required initial knowledge and skills of the PhD candidate

a) A degree in veterinary medicine
b) Experience in writing scientific publications in the field of veterinary medicine.
c) Knowledge of research principles, methodological approaches and ethical standards in science.
d) Ability to independently search for information, analyse problems, formulate conclusions and propose solutions.
e) Ability to interpret haematological and biochemical blood test results.
f) Interpretation of cardiac examination results in dogs.
g) Conducting and analysing ELISA test results.

1.6. Expected development of the PhD candidate's knowledge and skills

a) Development of knowledge and skills in the diagnosis and treatment of MMVD in dogs.
b) Development of knowledge and skills in the field of animal ageing.
c) Development of knowledge and skills in the field of clinical research in veterinary medicine.
d) Learning about telomere length testing procedures.
e) Learning about DNA methylation profiling procedures.