Theses of a doctoral (PhD) dissertation

CRITICAL ILLNESS-RELATED CORTICOSTEROID INSUFFICIENCY AND ASSESSMENT OF GLUCOCORTICOID RECEPTOR EXPRESSION IN PERIPHERAL WHITE BLOOD CELLS OF DOGS

dr. Csöndes Judit

Supervisors: Dr. Vajdovich Péter and Dr. Máthé Ákos

UNIVERSITY OF VETERINARY MEDICINE BUDAPEST

Veterinary Doctoral School

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1 Background and objectives

In my thesis entitled, *Assessment of Critical Illness-related Corticosteroid Insufficiency Syndrome and mRNA Expression of Glucocorticoid Receptor Isoforms in Peripheral Blood of Dogs*, I investigated the hypothesis, that the inadequate activation of hypothalamic-pituitary-adrenal (HPA)-axis ultimately results in relative hypocortisolaemia, moreover the target cells' inappropriate response to endogenous cortisol may decrease the chance of survival in critically ill dogs.

Septic and non-septic systemic inflammatory response syndrome (SIRS) is a common cause of hospital admission in dogs and similarly to human data, the mortality is as high as 21–68%. Critical Illness-related Corticosteroid Insufficiency (CIRCI) is a well-described medical entity in human beings and has also been reported in dogs. CIRCI is characterized by decreased cortisol production and/or glucocorticoid resistance at receptorial and/or post-receptorial level. Interestingly, decreased cortisol secretion does not necessarily mean absolute hypocortisolaemia, rather than inadequate glucocorticoid effect to alleviate ongoing inflammatory process.

Glucocorticoids are essential to maintain hemodynamic stability and to downregulate inflammatory signalling pathways during a critical illness. Glucocorticoids' genomic action depends on the glucocorticoid receptor (GR). Though the GR is encoded by a single gene (*NR3C1*), plenty of its isoforms have been already identified in many species. Critical illness results in activation of the HPA-axis, which might be accompanied by a peripheral adaptation in glucocorticoid sensitivity. According to studies the chance of survival is higher if the patient has lower serum cortisol level and/or elevated expression level of GR in peripheral blood. Increased expression of peripheral blood glucocorticoid receptor might be an adaptive mechanism for the increased need of anti-inflammatory effect of cortisol.

Early identification of critically ill patients with diminished cortisol response should be a priority in clinical practice in order to recognize a more severe disease course and/or poor disease outcome. As dogs serve as a model species for several inflammatory disorders of people, e.g. sepsis, rheumatoid arthritis, atopic dermatitis, and inflammatory bowel disease, we hoped that our findings may also contribute to the understanding of human glucocorticoid receptor signalling pathways.

In the first phase of the research our main goals were to assess the potential relationship between inflammatory biomarkers used in clinical setting (body temperature, total white blood cell count, presence of left shift on leukogram, serum albumin and C-reactive protein

concentration) and results of ACTH stimulation test (baseline and stimulated cortisol levels, ∆cortisol value) in critically ill dogs admitted to the Small Animal Hospital of the University of Veterinary Medicine Budapest . Additionally, we also investigated the predictive value of two clinical scoring systems: the SIRS-criteria (de Laforcade, 2009) and the fast version of Acute Patient Physiologic and Laboratory Evaluation ($APPLE_{fast}$ score) (Giunti et al, 2015) regarding disease outcome.

In the second part of the study we aimed to investigate whether, similarly to humans, SIRS would trigger alternative splicing of the canine ortholog of *NR3C1* gene. Potential cGR isoforms were examined by new-generation mRNA-Sequence. Furthermore, we designed a qPCR approach including isoform specific TaqMan probes and primers to quantify the mRNA expression of cGR transcript variants in peripheral blood samples from a cohort of healthy subjects and critically ill dogs. To evaluate gene expression levels the comparative threshold method (ddCt method) and relative quantification were used.

Finally, we assessed the correlation of various clinicopathological parameters and mRNA expression level of cGR transcript variants. Expression levels of glucocorticoid receptor isoforms were also evaluated in the light of disease outcome.

2 Summary

Firstly, adrenocortical reserve capacity and different inflammatory biomarkers were evaluated in the light of disease outcome. 50 critically ill dogs, admitted to the Small Animal Hospital of the University of Veterinary Medicine Budapest, were enrolled in the study between January 2014 and August 2016. Patients having pre-existing adrenal disease or receiving medications affecting the HPA-axis (e.g., glucocorticoids, progestins, major analgesics, azole antifungals) were automatically excluded. All subjects underwent a complete physical examination and venous blood sample was obtained for complete blood count and clinical chemistry panel within 24 hours after hospital admission. All patient received high standard of veterinary care during the hospitalization period.

Dogs classified as survivors were alive when discharged from the hospital. Subjects were classified as non-survivors if they died or were euthanized during the hospitalization period. Patients euthanized due to financial considerations were excluded. The owners of all enrolled dogs gave their written consent to the hospitalization and treatment. The Animal Welfare Committee of the University of Veterinary Medicine agreed to carry out this clinical study.

Using a logistic regression model increasing $APPLE_{fast}$ score was associated with a decreasing chance of survival ($p= 0.0420$). Application of SIRS criteria system was not helpful to predict the presence of systemic inflammatory response syndrome at the time of the hospital admission in our study population. None of the haematological or biochemical markers of inflammation were correlated significantly with disease outcome.

Noticeably, elevated basal ($OR = 9.71$, $p = 0.0135$) or stimulated ($OR = 3.69$, $p = 0.0311$) cortisol levels were associated with a higher chance of non-survival in the study population (Fisher's exact test), retrospectively. A value of $p < 0.05$ was considered significant for all tests.

Similarly, to previously published findings, none of the patients had hypocortisolaemia compared to the population-based reference interval. Additionally, our data indicated that pathologically higher basal and/or stimulated cortisol levels represent an exaggerated stress response in critically ill dogs, which was negatively associated with survival.

These controversial findings encouraged us further to investigate the expression of canine glucocorticoid receptor (cGR) at mRNA level and its changes in peripheral blood to evaluate the potential relationship between cGR expression and poor prognosis. For this purpose, we collected peripheral blood samples from 7 healthy and 26 critically ill dogs suffering from SIRS. One of the samples obtained from a dog with septic peritonitis was analysed by mRNA-Seq to identify the presumed splice variants of the *NR3C1* gene. For mRNA-Seq library construction, KAPA Stranded mRNA-Seq Kit for Illumina (Roche) was applied according to the manufacturer's protocol. The alignment of the retrieved sequences to the CanFam3.1

reference genome identified retention of intron 7 of the cGR mRNA. Analysis of read distribution between introns and coding exons showed that the ratio of *NR3C1* intron 7 to all *NR3C1* exons was an order of magnitude higher than the global intron to exon ratio suggesting that the alternative mRNA transcript is a bona fide splice variant.

In silico translation of the intron 7 retaining splice variant identified an early stop codon at the beginning of the intron 7 leading to a 680 amino acid long protein. The truncated protein is 101 amino acids shorter than the wildtype cGR and this shortening results in a partial loss of the ligand-binding domain. Considering the structural homology with the previously identified human and porcine GR-P splice variants the novel receptor subtype was reported as the canine GR-P isoform. cGR-P represents roughly 20% of the classical cGRα transcript pool based on the mRNA-Seq data.

The ubiquitous presence of cGRα and cGR-P in peripheral blood samples of healthy subjects and dogs suffering from SIRS were verified by TaqMan qPCR. The SIRS cohort was subdivided into survivor and non-survivor groups. Similar to our former findings, basal total cortisol level was significantly higher (p = 0.0336) in the non-survivor (median: 320.0 nmol/L; 41.6 - 1366.0 nmol/L) than in the survivor group (median: 131.0 nmol/L; (52.0 - 1131.0 nmol/L), retrospectively.

Furthermore, we analysed the mRNA expression levels between healthy, survivor and non-survivor groups. The median mRNA expression of cGRα was increased 5.6-fold (0.3 - 26.3-fold) in survivor dogs with SIRS compared to healthy controls (p = 0.0008), whereas its expression did not differ significantly between the control and non-survivor group (p = 0.5360). The median mRNA expression of the cGR-P splice variant was increased 9.8-fold (0.3 - 22.8-fold) in survivors compared to controls (p = 0.0005), and similarly to cGRα expression, cGR-P expression in the non-survivor cohort did not significantly differ from that of the healthy subjects (p = 0.3510). Interestingly, the ratio of median cGRα/cGR-P mRNA expression was not significantly different when the non-survivor cohort was compared to either the survivor (p = 0.4337) or the control group (p = 0.1738). A value of $p < 0.05$ was considered significant for all tests.

Compared to healthy dogs, neither of the two isoforms' expression were elevated in the non-survivor patients while a significant mRNA level elevation of cGRα and cGR-P were detected in samples of survivor SIRS patients. The strong correlation between cGRα and cGR-P mRNA expression in dogs with SIRS presumes a co-regulation of these two splice variants.

Critical illness amplifies the function of the HPA-axis which might be accompanied by hypercortisolaemia and increased glucocorticoid sensitivity of the target cells. On the one hand, elevated mRNA expression of cGRα and cGR-P in peripheral blood cells might be an adaptive mechanism for the increased demand of cortisol's anti-inflammatory effect. On the

other hand, critically ill patients with markedly elevated serum cortisol level might have lower cGRα and cGR-P expression due to ligand-induced receptor downregulation. Several human studies suggest that elevated serum cortisol level and/or decreased expression of GR in peripheral leukocytes correspond to a more severe disease course and poor outcome in patients with septic or non-septic SIRS.

3 New scientific results

1. Prevalence of Critical Illness-related Corticosteroid Insufficiency syndrome among critically ill dogs admitted to the Small Animal Hospital of University of Veterinary Medicine Budapest

According to the traditional interpretation of Critical Illness-related Corticosteroid Insufficiency Syndrome none of the critically ill subjects were suffering from hypocortisolaemia considering the population-based reference interval. On the contrary, those patients with elevated baseline and stimulated serum cortisol levels, had ten times and four times higher chance of non-survival, compared to those critically ill dogs which baseline and stimulated serum cortisol levels were within the reference range, retrospectively.

2. Description of a novel, previously not identified canine glucocorticoid receptor transcript variant (cGR-P) and demonstration of its ubiquitous presence in peripheral blood samples of healthy dogs and dogs with Systemic Inflammatory Response Syndrome

A novel previously not identified canine glucocorticoid receptor transcript variant (cGR-P) was detected in peripheral blood sample from a dog with septic peritonitis by new-generation RNA-Sequencing. The ubiquitous presence of cGR-P isoform was proved by TaqMan real-time PCR in peripheral blood samples of healthy dogs and dogs with Systemic Inflammatory Response Syndrome and found that this splice variant is highly abundant in the peripheral blood of healthy and sick dogs.

3. Evaluation of mRNA expression levels of glucocorticoid receptor α and P isoforms in peripheral blood samples of healthy dogs and dogs with Systemic Inflammatory Response Syndrome, in the latter case especially in the light of disease outcome

Real-time PCR analysis revealed that median cGRα and cGR-P mRNA expression levels were higher in dogs suffering from SIRS compared to the healthy control group. The median mRNA expression of cGRα was increased 5.6-fold in survivor dogs with SIRS compared to healthy controls, whereas its expression did not differ significantly between the control and non-survivor cohort. The median mRNA expression of the cGR-P splice variant was increased 9.8-fold in survivors compared to controls, and cGR-P expression in the non-survivor cohort did not significantly differ from that of the healthy controls.

Based on our results the total GR expression is more pertinent in assessment of the disease outcome, as the median mRNA expression of GRα/GR-P ratio did not differ significantly between survivor and non-survivor groups.

4 Publications

Publications in peer-reviewed scientific journal with impact factor

Csöndes Judit, Kiss Gergely, Máthé Ákos, Vajdovich Péter: **Hypocortisolaemia és glükokortikoid-rezisztencia kritikus állapotú kutyákban**. **Irodalmi összefoglaló,** Magyar Állatrovosok Lapja, 138. 681-693. 2016.

Judit Csöndes, Ibolya Fábián, Bernadett Szabó, Ákos Máthé and Péter Vajdovich: **Assessment of Adrenocortical Reserve Capacity and Inflammatory Parameters in Critically Ill Dogs**, Acta Veterinaria Hungarica, 65. 475-486, 2017.

Brigitta Margit Kállai, Judit Csöndes, Gergely Kiss, Lilla Bodrogi, Zsolt Rónai and Tamás Mészáros: **Restrained expression of canine glucocorticoid receptor splice variants α and P prognosticates fatal disease outcome in SIRS**, Scientific Reports, 11. 24505 2021.

Conference presentations

Judit Csöndes, Bernadett Szabó, Péter Vajdovich: **Clinical and laboratory evaluation of critically ill dogs suffering from inflammatory or neoplastic disorders** Poster presentation FECAVA-SEVC Congress Barcelona; October 15-17. 2015

Judit Csöndes, Ibolya Fábián, Ákos Máthé, Bernadett Szabó, Péter Vajdovich: **Assessment of Adrenocortical Reserve Capacity and Inflammatory Parameters in Critically Ill Dogs.** Free communication ESVCP Congress London; September 7-9. 2017

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