1st Human and Veterinary Crossover Symposium on Aldosterone

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First Human and Veterinary Crossover Symposium on Aldosterone

The contribution of aldosterone to heart failure was the focus of a recent symposium, hosted by Ceva Santé Animale. The principle of this symposium was to promote expertise exchanges between human and veterinary medicine. The programme included eminent cardiology researchers from around the world, both from the human and veterinary fields. You will find in this report a summary of the presentations and the discussions that took place, mixing state of the art science with the most recent clinical developments in man and animals.

We hope that you will both enjoy and benefit from the research compiled herein.

Sylvie Bourrelier and Emilie Guillot
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Speakers and chairs at the meeting, from left to right: Frédéric Jaisser, Nicolette Farman, Faiez Zannad, Bertram Pitt, Jonathan Elliott, Mark Oyama, Johann Bauersachs, Claudio Bussadori, John Bland and Robert Hamlin

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Nicolette Farman is Research Director (Senior Investigator) at INSERM (French National Institute of Health and Medical Research). She joined the public research body in 1978 (INSERM U2, Limeil Brévannes Hospital) and was a Board Member between 2001 and 2008.

She is a Member of the French Society of Nephrology, American Society of Nephrology, American Physiological Society and Honorary Member of the South-American Society of Nephrology. She is referee for several peer-review journals such as J. Am Soc. Nephrology, Am. J. Kidney Diseases, Endocrinology, Nature.

Nicolette Farman has organized four international congresses dealing with aldosterone in France and abroad. She was one of the pioneers in discovering the cardio-vascular effects of this hormone. She received the title of “Knight of National Order of Merit” and the Award of the Academy of Medicine in 2001. She is currently working alongside Dr Frédéric Jaisser at Unit 872 of INSERM.

ALDOSTERONE AND MINERALOCORTICOID RECEPTORS: EXPANDING VIEWS FROM THE KIDNEY TO THE CARDIOVASCULAR SYSTEM

The steroid hormone aldosterone plays a major role in the control of blood pressure and extracellular volume homeostasis. Aldosterone is synthesized in the glomerular zone of the adrenal cortex in response to hyperkalemia or sodium depletion, as the endpoint of activation of the renin-angiotensin system. Considerable work has been achieved to improve the understanding of the molecular and cellular events involved in renal aldosterone actions, in normal as well as in pathological situations.

It is well established that aldosterone stimulates renal sodium reabsorption and potassium excretion, an effect that can be blocked by administration of the mineralocorticoid receptor (MR) antagonist spironolactone. These effects are observed 1-2hrs after hormone administration, a delay required for hormonally-mediated activation of sodium transporters and regulation of transcription of target genes. Aldosterone binds to the MR, which is a ligand-dependent transcription factor that belongs to the nuclear receptor superfamily. Once translocated into the nucleus, hormone-receptor complexes bind to glucocorticoid response elements within the promoter region of early aldosterone-induced genes to modulate their transcription. This triggers an increase in the activity and number of sodium transporters or channels. In a typical target cell for aldosterone of the distal nephron, such as the renal collecting duct principal cell, the sodium of the tubular fluid enters the cell through amiloride-sensitive apical sodium channels, and it then extruded from the cell to the peritubular space by the Na-KATPase located in the basolateral membrane.

MR antagonists (spironolactone, eplerenone) prevent aldosterone binding and thus are useful therapeutic tools to prevent inappropriate MR activation. The MR has been cloned in 1987, and it was evidenced that the MR displays similar high affinity (in the nanomolar range) for aldosterone and glucocorticoid hormones, that are much more abundant in the plasma (100-1000 fold) than aldosterone. This should lead to permanent illicit occupancy of the MR by glucocorticoid hormones, inducing permanent maximal sodium retention, independent of plasma aldosterone levels. The MR protector enzyme 11ß hydroxysteroid dehydrogenase type 2 (11-HSD2, coexpressed with the MR in cells of the distal nephron) metabolizes circulating glucocorticoid hormones (cortisol in man, corticosterone in rodents) into inactive 11-dehydro derivatives (cortisone, 11-dehydrocorticosterone) with very low affinity for the MR.

More recently it became apparent that the MR is also expressed in the cardio-vascular system, together with low HSD2 and the nature of aldosterone action in these tissues is actively investigated, in normal as well as in pathological situations.
Beside the cardiovascular consequences of an altered functioning of the renin-angiotensin system leading to hypertension, direct cardiovascular effects of aldosterone have been evidenced. There are many data in favour of an involvement of aldosterone in cardiac remodelling and fibrosis. Exposure of uninephrectomized rats to aldosterone and high salt diet leads to cardiac fibrosis that can be prevented by spironolactone. Importantly, the RALES and the EPHESUS studies have demonstrated the beneficial effect of MR-antagonists (spironolactone, eplerenone) in large cohorts of patients with severe heart failure or following myocardial infarction. These initial observations have now opened a major new field to improve treatments of cardiac failure in patients. Major efforts are now made to elucidate aldosterone impacts on cardiovascular functions.

References

Frédéric Jaisser is Research Director at the Unit 872 of INSERM (Institut National de la santé et de la Recherche Médicale), the French public research body entirely dedicated to human health. He is Professor at the Faculty of Medicine of Reims where he coordinates different courses such as « Animal Models and Pathophysiological Mechanisms ».

He is MD, specialist in Nephrology and has a University degree in Biological and Medical Engineering. He joined INSERM in 1996. His fields of expertise are mainly renal and cardiovascular pathophysiology, development of transgenic animal models for pathophysiologic studies or human disease models. He is also the coordinator of several multicentric projects.

Frédéric Jaisser is an Editorial Board Member of Endocrinology and an expert for several other peer-review journals such as Circulation or Hypertension. He is currently the President of ESAC-France (European Section of the Aldosterone Council).

**OVEREXPRESSION OF THE MINERALOCORTICOID RECEPTOR: PATHOPHYSIOLOGICAL CONSEQUENCES**

**1- Mineralocorticoids and Cardiovascular diseases**

Aldosterone (Aldo) is the main regulator of sodium reabsorption \(^1\). The mineralocorticoid receptor (MR) is activated by Aldo binding and is targeted to the nucleus where it binds to Glucocorticoid (Gluco) Responsive Elements and triggers gene transcription. New targets of Aldo have been discovered in the last decade, that extends its actions to the cardiovascular (CV) system \(^2\). We have shown that MR is expressed in cardiomyocytes, coronary vessels, aorta and resistance arteries. MR is expressed in both endothelial and smooth muscle cells where its functions are still poorly understood \(^3\). Vascular-MR activation, as addressed in pharmacological experimental models or in human volunteers and patients with CV diseases, has been proposed to be linked to endothelial dysfunction and vascular wall remodeling \(^4,5\).

The RALES and EPHESUS clinical trials have demonstrated that the addition of MR antagonists to standard care markedly reduce the overall and cardiovascular mortality in patients with heart failure or in acute myocardial infarction complicated by left ventricular dysfunction, respectively \(^6,7\). Moreover, MR antagonism has been recently shown to be beneficial in diabetic nephropathy, as well as in chronic renal diseases \(^8\). The beneficial effects of MR antagonists in CV diseases are explained mainly by a decrease of cardiac fibrosis and an improvement of peripheral vascular function. However, direct effect on cardiac myocytes, endothelium and smooth muscle cells are still underscored.

Plasma Aldo levels are not correlated with the clinical response to MR antagonists. This may be explained by an increase in MR expression/MR activation in heart, vessel and kidney as reported in human diseases and experimental animal models. MR expression is increased in heart after myocardial infarction, heart failure and hypertension \(^8,10\). In the vessels MR expression is increased in arterioles of SHR rats \(^11\). MR activation (in absence of increased Aldo or MR expression) has also been proposed to be related to cellular oxidative stress.

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The signaling pathways involved in mineralocorticoid effects in CV diseases remain elusive because: 1) most of the proposed signaling pathways have been identified in cultured cells; 2) their identification using in vivo pharmacological approaches (aldosterone infusion, MR antagonism) is hampered by the pleiotropic effects of Aldo and MR activation, mixing primary events with the confounding secondary responses, 3) in vitro, the MR can bind with similar affinities both Aldo and Gluco. Thus, analyzing the specificity of Aldo/Gluco/ MR signaling pathways is fundamental to understand their pathophysiological roles in the CV system.

Very few genes have been reported to be modulated by MR activation in the endothelium, mainly based on...
short-term Aldo treatment of cultured cells 12. 13. 14. Activation of MAP kinases pathways has been reported in vascular smooth muscle cells, as well as induction of genes involved in vascular fibrosis, inflammation and calcifications (see for review 14). So far; biomarkers of MR activation have not been identified.

2- Conditional transgenic models: a clue to understand specific molecular and functional roles of MR activation in the heart and vessels

The profibrotic effect of aldosterone has been first identified in 1990 by Brilla and Weber in a pharmacological model combining uninephrectomy, high salt diet and infusion of aldosterone (or DOCA, a mineralocorticoid analogue) 15. Both right and left ventricles presented with interstitial remodelling suggesting that this was related to humoral disorders but not hemodynamic alterations due to high blood pressure. Indeed administration of a low dose of spironolactone, which was unable to prevent blood pressure increase, prevented cardiac and vascular remodelling as well as local inflammation 16-18. This confirmed that the deleterious effects were not related to hemodynamic factors but to local Aldo/MR activation.

Experimental models, such as non-targeted MR gene inactivation or pharmacological models (manipulating ligands or MR/GR antagonists) are complex to analyze. The pleiotropic effects of Aldo and Gluco as well as the large tissue distribution of their respective receptors make difficult to unravel their specific contributions to organ/cell pathophysiology. Inducible transgenic models make difficult to unravel their specific contributions to organ/cell pathophysiology. Inducible transgenic models allow spatio-temporal control of MR and GR expression, in selected target cells as well as precise tuning of receptor expression over time. The targeted approach rules out the possibility of secondary effects due to changes in renal ion homeostasis or to general Aldo/ Gluco-induced disturbances and allows investigation on the specific role of the MRGR in the cardiomyocytes or vessels 19. This segmental analysis requires in a second step an approach with pharmacological or constitutive, generalized gene manipulations.

Several double-transgenic mouse models with conditional, inducible manipulation of MR and GR expression have been generated and functionally analyzed in our laboratory. Such animal models provide tools that allowed to progress in the understanding of the steroid-mediated pathophysiology. This allows to address: i) Molecular and functional effects of MR in each target organ/cell type ii) Integrated in vivo responses permitting the analysis of both primary and secondary effects iii) Combination of genetic and pharmacological approaches to define specificity of the responses and of the identified pathways.

Pathophysiological role of MR/GR in the heart

1- Cardiac MR over-expression led to major electrophysiological abnormalities with prolonged ventricular repolarisation and spontaneous and triggered ventricular arrhythmias. This was associated with ion channel remodelling (patch-clamp) leading to a decrease in Ito K current and an increase in action potential duration and Ca transient amplitude. During embryogenesis and adulthood, mice exhibited a high rate of death prevented by the MR antagonist spironolactone 20.

2- A cardiac GR over-expressing model shows several phenotypic differences as compared to the MR model, with major conduction defects and a mild dilated cardiomyopathy, but no embryonic lethality. Ion channel remodelling also differed, indicating that specific pathways are distinctly regulated via MR or GR activation in the heart 21.

Pathophysiological role of MR in the vessels

In order to manipulate Aldo/Gluco and MR/GR signalling in the vessels, MR over-expression has been recently achieved in endothelial cells using an endothelial-specific transactivator strain. Increased MR activation in endothelial cells only is associated with increased blood pressure (independently of alterations in ion homeostasis), altered vascular reactivity as estimated ex vivo in mesentenic arteries and altered in vivo blood pressure response to vasoconstrictors like AngII and ET1.

Identification of specific gene targets of the MR

Differential screening of gene targets specifically modulated upon MR activation but not GR activation was done in the heart of the MR/GR conditional models. Using transcriptome analysis done in heart samples from mice with conditional overexpression of either MR or GR in the cardiomyocytes only, we analyzed differential expression of 10 000 mouse genes (using MWG microarrays) as well as 6000 genes selected for their putative implication in heart diseases (these genes were selected based on their expression in cardiac tissue affected by various pathologies). Some of the genes overlapped between the two arrays. We were able to identify networks of genes specifically up- or down- regulated in the MR or GR model. Several candidates were selected from these results and validated in individual mice at different time points after MRGR expression. About 15 genes that were up- or down- regulated in the MR model (as compared to control mice and GR mice) were validated.

Some of them have been proposed as putative biomarkers of MR activation since they encodes secreted protein that have been identified in the plasma of these transgenic models as well as in a rat heart failure model. Interestingly, pharmacological MR antagonism normalized the increased plasma level of these biomarkers.

Taken together; our data indicate that:

-Our unique models are powerful tools to identify and validate biomarkers of MR activation.

-Such biomarkers of MR activation may be particularly useful in human and canine patients with heart failure, human patients with infection, metabolic syndrome (including diabetes), as companion diagnostic in order to adapt the treatment on an individual basis. Indeed we have shown that one of these markers is also expressed in the dog but its relevance in canine heart failure remains to be studied.
OVEREXPRESSION OF THE MINERALOCORTICOID RECEPTOR: PATHOPHYSIOLOGICAL CONSEQUENCES

References


ANSWERS FROM: NICOLETTE FARMAN (NF) AND FRÉDÉRIC JAISSER (FJ)

Question (Chair) - There is interest in non-genomic effects of mineralocorticoid receptors (MR) – are these of physiological significance, or simply a red herring?

Answer (NF) - Aldosterone acts rapidly in the cytoplasm to modulate cell function in cell cultures, for example on calcium and pH, suggesting such effect but these have not been proven in living animals or humans. So we do not yet know the answer.

Question (Delegate) - How much do we know about MR in fibroblasts? Does activation have an effect on the amount of collagen, or the cross-linking or quality of the collagen?

Answer (FJ) - High doses of aldosterone have been shown to induce collagen synthesis in fibroblasts in vitro, but it is not known if MR is expressed in fibroblasts in vivo. Aldosterone has been shown to bind to glucocorticoid receptors (GR) in many studies and high doses of MR antagonists appear to block GR activity. This could, therefore, explain how aldosterone induces collagen synthesis. Current consensus is, however, that fibrosis is not necessarily a direct effect of MR activation and that other factors, e.g. oxidative stress, may have a secondary effect on remodelling and vascular changes.

Question (Delegate) - How much is known of the different MR genetic variants? And does treatment with MR antagonists differ between these genotypes?

Answer (NF) - There is a mutation that affects the MR - a single amino acid mutation - that changes the activation of the MR-aldosterone ligand so that progesterone, for instance, changes from an antagonist to an agonist. Also, spironolactone becomes an agonist and children with this mutation treated with spironolactone actually develop hypertension. However, little is known about other mutations and how they differ in their sensitivity to antagonists.
Question (Speaker: Robert Hamlin, USA) - The development of drugs that serve as ligands for many receptors and which have minimal side effects is desirable. Would it, therefore, be better to develop drugs that work further downstream, e.g. on calmodulin, and focus on specific end points?
Answer (FJ) - Yes, but will it affect the pathology? I am not sure that such an approach would be specific enough.

Question (Chair) - How are we going to develop MR antagonists with tissue-specific effects?
Answer (NF) - I don’t know. If I did I would be very rich and not here at the moment! Can we identify co-activators or co-repressors that are more specific for MR than other receptors? Or tissue-specific receptors? These are excellent targets for our research and are the future.
Answer (FJ) - You need to bear in mind that antagonists block receptors in certain configurations and so, dependent upon the cofactor, the structure of the receptor is different.

Question (Delegate) - Can we assume that all dog breeds would have the same MR expression in cardiomyocytes? After all there is evidence in rats that different levels of MR expression occur between breeds.
Answer (NF) - In principle it is possible that the expression of MR in the hearts of dogs would mimic that seen in other species. It is not difficult to do the research to find out.

Comment (Chair) - Pharmacogenomics is a huge area and one in which we are way behind in veterinary medicine. Especially since dogs represent such a wide range of breeds with hugely different genetics.

Question (Delegate) - It is fascinating that, in the RALES study, the effects of spironolactone were independent of measured circulating concentrations of aldosterone. Why is this? Are there factors that alter the downstream response to MR, or is there more aldosterone production and intracellular action that is not reflected in measurements from blood samples?
Answer (FJ) - There is debate and several possibilities: 1) is there local production of aldosterone, i.e. in cardiomyocytes? 2) Is there increased production of MR? 3) Is there increased capacity of MR to be activated by the same level of aldosterone, or is there more co-activator produced or available? 4) Is the ligand not aldosterone but glucocorticoid in this clinical setting? Due to oxidative stress, there is a change in the ability of MR to be activated by glucocorticoids. There is a correlation between glucocorticoid levels and survival from heart disease, which may be due to activation of GR. There is, therefore, no clear explanation.
Johann Bauersachs is an Associate Professor at the University Hospital of Würzburg (Germany) in the Division of Cardiology. He has Board Certifications in Internal Medicine Cardiology and Critical Care. He received two Awards from the German Society of Cardiology (2001, 2004) and more recently the Parmley-Award of the American College of Cardiology in 2007.

His current research topics are healing and remodeling after myocardial infarction; cardiac ischemia- and reperfusion injury; cardiac regeneration by progenitor cells; endothelial progenitor cells, endothelial dysfunction and platelet activation in coronary artery disease, myocardial infarction, hypertension and diabetes mellitus.

He is member of the Editorial Board of Cardiovascular Research, European Journal of Clinical Investigation, and Basic Research in Cardiology. Johann Bauersachs is the President of the European Section of the Aldosterone Council (ESAC) in Germany since 2006.

MINERALOCORTICOID RECEPTOR BLOCKADE: \ HOW EXPERIMENTAL EVIDENCE SUPPORTS THE CLINICAL BENEFIT

Activation of the renin-angiotensin-aldosterone system plays an important role in the pathogenesis of cardiovascular disease. High levels of aldosterone impair cardiac and vascular function and predict mortality risk of patients with acute myocardial infarction or heart failure. Patients with primary hyperaldosteronism display a higher incidence of myocardial infarction and stroke. Large clinical trials (RALES, EPHESUS) have shown mineralocorticoid receptor (MR) blockade to decrease mortality in heart failure 1,2.

In contrast to the classical notion that mineralocorticoids were only involved in body electrolyte and water homeostasis mediated by the kidney, aldosterone exerts important direct (patho)physiological effects on the cardiovascular system. While the extra-adrenal generation of aldosterone from heart and vessels appears to be of minor importance, MR expression has been repeatedly documented in cardiovascular cells. Aldosterone targets MR on cardiomyocytes, cardiac fibroblasts, endothelial cells and smooth muscle cells leading to cardiac hypertrophy, fibrosis and vascular injury. MR expression in the heart is increased in heart failure explaining detrimental effects of aldosterone even in the absence of markedly elevated circulating levels of aldosterone. Under certain pathophysiological circumstances, also glucocorticoids may act as agonists on the MR. Upon binding to the MR aldosterone increases cardiomyocyte as well as vascular endothelial and smooth muscle cell reactive oxygen species formation 3,4,5.

In dogs with chronic heart failure induced by microembolization, long-term treatment with the MR blocker eplerenone attenuated progressive left ventricular (LV) dysfunction as well as LV dilatation, hypertrophy and fibrosis. In dogs with heart failure induced by rapid pacing, spironolactone reduced atrial structural and electric remodelling, associated with significantly less and shorter episodes of atrial fibrillation 6,7.

Long-term MR blockade with eplerenone and angiotensin converting enzyme (ACE) inhibition alone or in combination were studied in rats with heart failure after extensive myocardial infarction. Like ACE inhibition, eplerenone attenuated LV filling pressure and volume. Combination therapy significantly improved LV systolic and diastolic function, and reduced LV end-diastolic volume. In parallel, molecular alterations such as changes in collagen expression or myosin heavy chain isoforms were reversed by combination therapy 8.

In organ baths studies, the effect of MR blockade on endothelial function was assessed using phenylephrine-preconstricted aortic rings from rats with chronic heart failure. Long-term treatment with spironolactone in addition to
ACE inhibition normalized the acetylcholine-induced nitric oxide-mediated relaxation which was significantly attenuated in rats treated with placebo, and prevented the increase in superoxide anion formation. MR blockade also attenuated the platelet activation observed in rats with heart failure, an effect which may also translate into reduction of thrombo-embolic events in patients with heart failure.\(^{9,10}\)

To clarify that indeed direct effects in the heart mediate beneficial actions of MR blockade, we investigated LV remodeling after myocardial infarction in mice with cardiomyocyte-specific deletion of the MR gene (MR\(^{-/-}\)) which were generated using a conditional MR allele MRRox in combination with a transgene expressing Cre recombinase under control of the myosin light chain (MLC2a) gene promoter. Sham-operated wildtype and MRRox\(^{-/-}\) mice showed similar cardiac morphology and function. LV function was preserved and LV dilatation was attenuated in the MR\(^{-/-}\) mice with chronic heart failure compared to wildtype animals. This was associated with reduced superoxide formation, myocyte cross-sectional area and collagen accumulation in the surviving myocardium. Thus, myocyte-specific deletion of the MR gene protects against adverse cardiac remodeling and contractile dysfunction in ischemic heart failure, suggesting that clinical benefits of MR blockade in patients with heart failure may be mediated at least in part via a cardiomyocyte-dependent mechanism.

As in the EPHEsus study in patients with acute myocardial infarction complicated by heart failure there was a striking reduction of mortality by eplerenone as early as 30 days after randomisation, we examined whether MR antagonism promotes healing of the infarcted myocardium. Starting immediately after coronary ligation, rats were treated with the selective MR antagonist eplerenone or placebo. At seven days, eplerenone therapy versus placebo significantly reduced thinning and dilatation of the infarcted wall, improved left ventricular LV function and enhanced neovessel formation in the injured myocardium.\(^{11,12}\)

As bone marrow derived endothelial progenitor cells (EPC) play an important role in endothelial repair and angiogenesis, we hypothesized that hyperaldosteronism impairs EPC function and vasculogenesis capacity in mice and humans. Treatment of human EPC with aldosterone induced translocation of the MR and impaired multiple cellular functions of EPC, such as differentiation, migration, and proliferation in vitro. Aldosterone protein kinase A-dependently increased reactive oxygen species formation in EPC. Aldosterone infusion in mice impaired EPC function and vasculogenesis capacity in a MR-dependent manner. EPC from patients with primary hyperaldosteronism compared to age-matched controls displayed reduced migratory potential. MR blockade in patients with hyperaldosteronism improved EPC as well as endothelial function. Thus, normalization of EPC function may represent a novel mechanism contributing to the beneficial effects of MR blockade in heart failure and other cardiovascular diseases.

In the Würzburg heart failure registry including consecutive heart failure patients with either systolic or diastolic dysfunction and structural remodeling of atrium in congestive heart failure dogs. Chin Med J (Engl) 121(1): 38-42.

In conclusion, there is ample evidence from experimental and clinical trials that mineralocorticoid receptor blockade reduces mortality and morbidity in heart failure.

Low dosages of spironolactone and eplerenone devoid of substantial diuretic effects have been used in the large randomised clinical trials. Experimental evidence supports the concept that direct actions of MR blockade in the heart and vasculature mediate at least part of the beneficial effects on left ventricular remodelling.

Selected References

**ANSWERS FROM:**

**JOHANN BAUERSACHS**

**MD - Associate Professor and Consultant in Cardiology and Critical Care, University Hospital, Würzburg, Germany**

**Question (Chair) -** There is clearly evidence in the literature concerning acute models of myocardial infarction, some of which involve dogs, but most of the audience here are interested in chronic heart failure. Which of the experimental models are predictive of benefit in chronic heart failure?

Answer - The mouse and rat myocardial infarction models are not, in fact, models of acute disease. Heart failure develops within several weeks and so they are viewed as models of chronic heart failure. For example, much of the development work on ACE inhibitors was done using these models during the 1980s and early 90s. To be efficient as models of heart failure, we make large infarctions and do not start treatment too early. The dog model in which heart failure is induced by ventricular pacing is not often used now because it is not necessarily viewed as a good model of human heart failure.

**Question (Speaker: Robert Hamlin, USA) -** How are you convinced that the relation between mineralocorticoids and glucocorticoids (in determining survival for humans with heart failure) is a cause or effect of heart failure?

Answer - There are good data from treatment studies and MR knock-out mice that the effects of MR activation develop over time, and that it is early as well as late stage MR activation that contributes to the pathology. There is also a dose effect in that less MR activation is seen in less severe heart failure.

**Question (Speaker: Robert Hamlin, USA) -** Do you believe that the mechanism by which heart failure occurs is important in determining the response to therapy? Does the mechanism of induction determine the response to treatment?

Answer - There appears to be no clinical distinction in terms of symptoms despite the different underlying pathologies, but the effects of drugs are important and different. The best treatments are those that work in multiple models of heart failure. Spironolactone, for example, does work in multiple models.

**Question (Delegate) -** I would like to know how diffuse are the lesions caused by aldosterone over-activation? Are the lesions concentrated around the main infarction? How about lesions in dilated cardiomyopathy, for instance? Are these in the left or right ventricles?

Answer - Lesions are not confined to the infarct areas. We have looked at the surviving myocardium and found marked collagen accumulation and seen effects of MR blockers. It is clear that spironolactone is of clinical benefit in right-sided heart failure, for example in patients with secondary hyperaldosteronism. There has been no investigation of fibrosis in humans with right-sided heart failure for the reason that it is difficult to obtain tissue. I would expect to see less fibrosis in the right ventricle where the right-sided failure is secondary to left-sided failure or lung disease.

**Comment (Speaker: Claudio Bussadori, Italy) -** The models discussed are good for looking at systolic dysfunction in the veterinary field, but there is no real model of chronic volume overload due to mitral regurgitation, especially in advancing age. If we are really doing something with spironolactone, we perhaps need myocardial biopsies. To compare experimental mitral valve insufficiency with spontaneous disease is completely wrong, as the experimental models, which involve cutting the chordate tendinae, result in acute mitral valve insufficiency. So the experimental model should, in fact, be the disease itself.

**Question (Chair) -** Of the human studies involving patients with non-ischaemic heart disease, which best relate to veterinary medicine?

Answer - These points are well taken and we will see the dog data later. Similar mechanisms work in different cell types in chronic heart failure because of, for example, this ‘foetal switch’. So, to some extent, they are all similar at the end stage regardless of how disease has been induced. The situation is, however, completely different when we want to prevent progression in the earlier stages.

**Question (Speaker: Claudio Bussadori, Italy) -** Sudden death is a real risk soon after acute myocardial infarction due to ventricular fibrillation. But this does not happen in chronic mitral valve regurgitation. One may talk about right ventricular failure due to chronic cor pulmonale, but this is not common in dogs compared with humans. Emphysema is rare in dogs because they do not live long enough for this to develop. They probably do develop right ventricular dysfunction because of a dilated left ventricle and displacement of the septum. In these cases the diuretic effect of spironolactone is important and therapeutic benefit is not simply due to its antifibrotic effect.

Answer - The best evidence that spironolactone works in humans comes from the RALES study and here there was no difference between patients with ischaemic or non-ischaemic disease such as dilated cardiomyopathy. A lot of these patients also had mitral insufficiency and in these cases, spironolactone still worked extremely well.
Robert Hamlin is the Stanton Youngberg Professor of Physiology/Pharmacology, a Professor of Biomedical Engineering and a Professor at the Davis Heart and Lung Research Institute, Ohio State University. He is Scientific Director of QTest Labs. His research interests are safety pharmacology, comparative electrocardiography, cardio-pulmonary interactions in the genesis of signs and symptoms, models of heart failure and arrhythmia, cardiac resynchronization therapy, and pulmonary mechanics.

He was Past President and Chairman of the Board of the ACVIM, Past President of the Central Ohio Heart Association, recipient of an NIH Career Development Award, and received Distinguished Professor and Distinguished Lecture Awards at The Ohio State University. Dr. Hamlin has published over 300 refereed research publications and 18 textbook chapters on cardiovascular medicine, and cardiovascular physiology and pharmacology.

THE PROXIMATE CAUSE OF HEART FAILURE:
MODELS, REMODELING, AND RE-REMODELING

Definition and Proximate Cause of Heart failure:

Heart failure is a set of signs and symptoms—a syndrome—produced by a pathological interaction among the heart, blood vessels, and their neuroendocrine control. A failing heart is characterized by: (1) reduced rate of cycling of heavy meromyosin heads (the fundamental units of contraction), (2) reduced rate of hydrolysis of ATP to ADP and release of energy, (3) reduced slope (Emax) of the connection of end-systolic points of pressure-volume loops obtained at varying preloads, and (4) decreased force or rate of force development for a given preload. Heart failure has multiple potential causes including valvular and/or myocardial disease, infection or toxicity, but most possess in common an overload of pressure and/or volume which may alter calcium kinetics (release and binding), depolarization and/or repolarization, energy transformation and/or availability, or alignment of contractile elements. The pathway of calcium kinetics is as follows:

(1) Abnormal release of calcium from the sarcoplasmic reticulum through the ryanodine release channel.
(2) The abnormal release is due to depletion of calcium in the sarcoplasmic reticulum due to calcium leaks in diastole, or to abnormal function of the ryanodine release channel.
(3) The deficiency of sarcoplasmic calcium is due to faulty entry into the sarcoplasmic reticulum through the SERCA++/ATPase pump and abnormal exit of calcium from the cytosol via the up-regulated Na+/Ca++ exchanger. Deficiency of ATP is not the proximate cause of heart failure, rather there is inadequate shuttling of ATP to the contractile elements.

Models of heart failure:

Models of heart failure are developed to study the genesis of signs and/or symptoms (i.e., pathophysiology), and to investigate therapeutic interventions. Models are classified by etiology: genetic modification (e.g., tropomodulin over-expression), toxic (e.g., cobalt, saponin), infectious (e.g., Trypomosoma cruzi), ischemic (e.g., coronary embolization), electrophysiological (e.g., heart block, rapid pacing), electrical ablation (e.g., transventricular shock), drug-induced (e.g., doxorubicin), pressure overload (e.g., aortic/pulmonic stenosis), volume overload (e.g., AV shunt, mitral/aortic regurgitation).

The vast majority of dogs presented with signs of heart disease are affected by mitral regurgitation, and are small breed, chondrodystrophic, aged dogs which may have multiple concurrent degenerative diseases (e.g., pulmonary fibrosis, chronic bronchitis, microscopic intramural...
myocardial infarction, chronic interstitial nephritis, lenticular sclerosis, periodontitis). The “Perfect Model” should be produced rapidly, inexpensively, have a high yield, **mimic the natural state**, safe for the investigator, uniform in severity, controllable, stable, humane; and be acceptable by the academic community, pharmaceutical industry, and drug regulatory agencies. Although acute rupture of either the mitral valvules or chordae tendinae mimics acute mitral regurgitation, the disease as observed in the clinic is mimicked better by adding rapid-pacing induced heart failure. The ultimate value of a model is determined by its predictive value (sensitivity and specificity) for providing scientific knowledge which can be extrapolated to the target species. The ultimate value of a model is determined by its predictive value (sensitivity and specificity) for providing scientific knowledge which can be extrapolated to the target species.

**Remodeling:**

Remodeling is a “catch-all” term for structural, biochemical, electrophysiological, physico-chemical, and genetic changes that occur in an organism that alters the quality and/or duration of life. Adaptive (physiologic) remodeling translates to increased quality and/or duration of life whereas maladaptive (pathologic) remodeling decreases quality and/or duration of life. Mal-adaptive remodeling often arises from chronic hemodynamic loading and/or neurohumoral activation. Examples of adaptive remodeling are the physiologic responses to exercise (athletes) or that which occurs with pregnancy. Adaptive remodeling is characterized by vascular dilatation, a brief increase in activation of renin-angiotensin- sympathetic nervous activity, and lengthening of action potential duration. Adaptive remodeling may transition from a compensatory, physiologic process to a maladaptive one.

**Reverse remodeling:**

Reverse remodeling, “re-remodeling”, refers to the return towards normal cardiac structure or function. Whereas re-remodeling may occur spontaneously after resolution (e.g., viral myocarditis), withdrawal (e.g., cisapride) or correction (e.g., pulmonic stenosis) of etiological agents, it occurs most frequently in response to active therapy (e.g., surgical closure of patent ductus arteriosus, ventricular assist device, cardiac resynchronization, stem cell transplantation, combination of ACE inhibitor and spironolactone). Re-remodeling is assessed using many of the same indices by which remodeling is determined and quantified: ventricular volumes, ejection fraction, sphericity index, myocardial dysynchrony, arrhythmias, and neuroendocrine activation. Agents known to promote re-remodeling are: ACE inhibitors, aldosterone inhibitors, AT1-antagonists, ß-blockers, blockers of sarcolemma Na+/H+ exchanger, and possibly dobutamine. Studies from one laboratory suggested that ACE inhibitor-increase in bradykinin in the left ventricle actually accelerated matrix loss and therefore was ineffective in treating rats with volume overload due to AV shunts. However the same group found that ß-receptor blockers protected ventricular function in dogs with iatrogenic mitral regurgitation, supporting the well-known beneficial effect of ß-receptor blockers. Since angiotension-II promotes release and decrease reuptake of NE, it is inexplicable why ACE inhibitors which decrease angiotensin-II should not produce a similar benefit.

phases of volume overload was in turn protective. Therefore they believe that angiotensin-II is important in the remodeling process and should be attenuated pharmacologically, but imply that ACE inhibition did not attenuate angiotensin-II production or activity, or possibly that, contrary to the putative beneficial vasodilatory activity of bradykinin, the increase in bradykinin counteracts the benefits of decreased angiotensin-II production or activity. They also showed that ß-receptor blockers protected ventricular function in dogs with iatrogenic mitral regurgitation, supporting the well-known beneficial effect of ß-receptor blockers. Since angiotension-II promotes release and decrease reuptake of NE, it is inexplicable why ACE inhibitors which decrease angiotensin-II should not produce a similar benefit.
References


ANSWERS FROM:

ROBERT HAMLIN

DVM, PhD, DipACVIM – Stanton Youberg Professor of Physiology/Pharmacology, Professor Biomedical Engineering, Professor Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio, USA

Question (Chair) - The kidney is clearly important in heart failure and there is a relationship between renal disease and heart disease in humans. Do you believe that renal disease plays a role in heart disease in veterinary patients?

Answer - Knowing the effects of ACE inhibitors on the heart, I am very enthusiastic about these drugs. My renal friends say that the best thing in their field in the last 20 years is ACE inhibitors. These drugs clearly have value in the treatment of heart and kidney disease, and may be useful in multiple diseases. Specific to your question, one aspect to heart failure is abnormal renal function. We understand the mechanisms by which this occurs and also understand that ACE inhibitors, spironolactone and, less so, diuretics should be useful. Let’s consider diuretic more. There is no argument that if you want to reduce pulmonary oedema there is nothing better than diuretics. But humans do not do as well on high doses of diuretics, so it is important to assess the interaction of the heart, lungs and kidneys when discussing pharmacological approaches.

Question (Delegate) - On your last slide you listed the three active agents that you like (ACE inhibitors, mineralocorticoid receptor antagonists, and β-blockers). Would you consider drugs that improve calcium cycling like pimobendan or levosimendan do belong to that list?

Answer - This is very difficult. There is no argument that levosimendan and pimobendan have beneficial effects in the veterinary clinic, but there is a group within the USA that has killed the idea that introtropic agents are beneficial. The issue is whether our responsibility is to the patient or the client. Regulatory agencies and academic bodies should ask whether drugs simply lengthen life, or change the quality of those. Those use pimobendan know it improves quality of life. They have been shown to reduce lifespan in humans. But they improve life in animals. I doubt that a positive inotrop will ever be approved in human medicine. The reason that I like calcium modifying agents is not necessarily because of their inotropic effects but rather because of their lusitropic actions.

Question (Delegate) - There are several works relating to aldosterone receptor blockers in dogs which show that they do not prevent an increase in ventricular size or systolic dysfunction, but do improve neuroendocrine and electrophysiological function. Is this how these drugs improve cardiac function and outcome?

Answer - Absolutely. I believe that the beneficial effects of these drugs are not an acute phenomenon; it takes a while to exert an effect due to interactions rather than immediate direct effects on the heart and blood vessels. I am not disturbed by the absence of an acute effect. But I do expect a chronic effect, which is difficult to analyse. Evidence will, I believe, show that attacking the neuroendocrine basis in veterinary medicine will accrue the same benefits as in humans.
Bertram Pitt is Professor of Medicine Emeritus at the University of Michigan School of Medicine. He is diplomate of the American Board of Internal Medicine and of the American Board of Cardiology.

He is a member of several professional societies such as the American College of Cardiology, the American Society for Clinical Investigation, the American Physiological Society – Circulation Group, the American Federation for Clinical Research and the American Heart Association. He has received awards like the Forest Dewey Dodrill Award for Excellence in 2001 and the James B. Herrick Award in 2005 (both from the American Heart Association).

He has published more than 500 papers in the most important peer-reviewed journals dealing with cardiovascular diseases (Circulation, American Heart Journal, Journal of the American College of Cardiology, Hypertension, European Heart Journal...). He is the first author of RALES and EPHESUS and he is currently leading large scale trials investigating the clinical benefit of mineralocorticoid receptor blockade in cardiac patients.

Mineralocorticoid receptor blockade (MRB) is emerging as an important component of the therapeutic approach to heart failure (HF). The results of the RALES trial 1 showing a 30% reduction in all cause mortality in patients with chronic severe HF (NYHA class III-IV) due to systolic left ventricular dysfunction (SLVD) randomized to the MRB spironolactone supported by the results of the EPHESUS trial 2 in patients with HF and SLVD post myocardial infarction randomized to the MRB eplerenone has led to a class 1 indication for MRB in both European and US guidelines. The benefits of MRB on mortality in HF appear to be in addition to standard therapy including an angiotensin converting enzyme inhibitor (ACE-I) or angiotensin receptor blocker (ARB), a beta adrenergic receptor blocker (BB), a loop diuretic and digoxin.

Spironolactone at 12.5-50 mg/day has been shown to be effective in reducing mortality in the RALES trial. This beneficial effect has been shown to be related to a reduction in myocardial and vascular inflammation, fibrosis, and hypertrophy as well as to an improvement in nitric oxide availability. The effectiveness of MRB in HF can in part be attributed to the finding that MR expression is increased in HF 3. Activation of the MR, whether by aldosterone or cortisol 4, has been shown to result in an up regulation of myocardial calcium channel expression, electrical remodeling of the myocardium, and a propensity toward sudden cardiac death as well ventricular remodeling and death due to progressive HF.

Elevated levels of both plasma aldosterone and cortisol have been shown to predict an increase in mortality and morbidity in patients with chronic HF 5. Of interest is the recent finding that plasma aldosterone levels in the upper tertile, but within normal limits, are predictive of an increase in mortality and morbidity in patients post myocardial infarction independent of the presence of HF, suggesting an important role of MRB in patients post myocardial infarction without HF or SLVD 6.

MRB is currently being evaluated in patients with NYHA Class II HF due to SLVD in the EMPHASIS-HF trial 7 and in patients with HF and a normal left ventricular ejection fraction (HFNEP) (Diastolic HF) in the TOPCAT trial 8. In view of the finding that MRB prevents myocardial and vascular fibrosis and hypertrophy it can be postulated that in the future MRB will play an even more important role in the prevention of HF in patients with essential hypertension and in the treatment of the entire spectrum of HF.
**References**


**Question Session**

**Bertram Pitt**
MD – Professor of Medicine Emeritus, University of Michigan School of Medicine, Michigan, USA

**Question (Chair) - One of the key differences in dogs is that, in European markets, a lower dose of spironolactone is used than in the USA. Have you observed a dose-dependent effect in RALES and your other studies?**

**Answer – I am nervous about the dose of spironolactone, especially when it is in the region of 100-200 mg/day. At the start of RALES we performed a dose-response study, looking at 12.5-100 mg/day, and found a marked increase in hyperkalaemia at doses in excess of 50 mg/day. Since we were concerned about drop-outs from the study we settled on a dose of 25 mg/day. There is benefit from higher doses but you must monitor safety by measuring plasma potassium concentrations, especially when doses are 100-200 mg/day. There is little diuretic effect below 100 mg/day and we are often using 15-25 mg once daily or 25 mg every second day.**

**Question (Speaker: Robert Hamlin, USA) - Comparing the number of persons or animals in heart failure with the number at risk or in early stages, is there an argument for instituting treatment very early in the disease?**

**Answer – Our goal should be to prevent heart failure in humans, but it will take 10-15,000 patients and several hundred million dollars to perform the requisite clinical studies, and so it is unlikely that this research will be done.**

**Question (Chair) - Many veterinary cardiologists are aware of the Alabama group’s model looking at acute mitral regurgitation induced by chordae rupture and which has shown that, at least in this model, early treatment with an ACE inhibitor prevents fibrosis from occurring and accelerates the amount of eccentric hypertrophy. Is there any sort of similar caution in too much neurohormonal blockade too early in human medicine?**

**Answer – It has not been examined so far in humans with chronic mitral regurgitation. Perhaps it is something we should go back and look at. I would not use an acute chordae rupture model since the disease entity is not adequately challenging. In chronic mitral regurgitation, the clues that we know about in human medicine suggest that ACE inhibitors would be beneficial.**

*Dell’Italia’s team, Department of Medicine, Division of Cardiology, University of Alabama at Birmingham, Birmingham, Alabama, and Dillon’s team, Auburn University College of Veterinary Medicine, Auburn, Alabama.*
Comment (Speaker: Johann Bauersachs, Germany) - In the CONSENSUS II study, where ACE inhibitors did not work, men experienced greater mortality in an acute decompensation situation, suggesting that ACE inhibitors should not be used in this situation.

Comment (Chair) - Your point is very well taken. For veterinary cardiologists, we have always looked at that models somewhat suspiciously. But we looked at that model because there’s really nothing else out there in the literature that comes close to approximating what we see in the chronic situation.

Comment (Delegate) - What the Alabama’s group has been able to show in its experimental dogs with chordae sectioning is a kind of basic fundamental process showing how a heart dilates and hypertrophies. It is also important to remember that the category of dogs we see in the clinic are old. They have vascular changes which has been convincingly shown by Torkel Falk and Lennart Jönsson. It is a reality and that’s something not seen in these young experimental dogs. There are probably additional layers than just the fundamental pathophysiology that come into play on this. These vascular changes do promote a replacement fibrosis that will occur close to the vessels in the myocardium.

Question (Chair) - Is there any difference between eplerenone and spironolactone?
Answer – I have asked Pfizer many times to do a comparison. There have been a few clinical comparisons and lots of experimental work. These show that their efficacy is similar. But their safety is different due to the risk of gynaecomastia in men and premenstrual problems in women being treated with spironolactone. In primary hyperaldosteronism, spironolactone looks better. Eplerenone has a shorter half life, which might make it safer.

Question (Delegate) - If you stratified patients according to renal function, would you see any differences in efficacy of MR antagonists?
Answer – There would be difference in safety, with patients having a GFR <60 being more at risk of hyperkalaemia. There may be an efficacy difference if the GFR is less than 50 or 30, but there is little evidence of spironolactone efficacy in these patients probably because of risk of hyperkalaemia. That’s about all I can say; there simply isn’t yet a firm answer to your question.
Claudio Bussadori is Director of the Gran Sasso Veterinary Clinic and Researcher at the paediatric cardiology department of S.Donato Hospital in S.Donato Milanese. He graduated as a Medical Doctor in 2001, thus becoming a human physician in addition to veterinary surgeon. He is Director of the ECVIM (European College of Veterinary Internal Medicine, Cardiology) Residency programme in his clinic and he also gives seminars lecture on congenital heart diseases at Faculty of Medicine, University of Milan. He was Vice President of the ECVIM between 1993 and 1999, ESVC President from 1997 to 1999. He has been an Honorary Member of the ESVC Board since 2002. His main fields of interest are diagnosis, epidemiology and interventional treatment of congenital heart diseases in dogs; echocardiography and pathophysiology of congenital cardiac diseases in humans and application of new echocardiographic technologies (2D Strain and 3D echocardiography) in human and veterinary cardiology.

Efficacy of Spironolactone in Dogs with Naturally Occurring Myxomatous Mitral Valve Disease

1. Introduction

In the RALES study (Randomized ALdactone Evaluation Study) a 31% reduction in the risk of mortality (cardiac causes) in patients receiving Spironolactone led to the premature termination of this study for ethical reasons. Aldosterone is thought to be similarly involved in the pathophysiology of canine heart failure (HF), hence clinical field studies were conducted to test whether Spironolactone would also be beneficial in dogs. The aim of these trials was to assess the effect of Spironolactone therapy on the risk of sudden cardiac death, euthanasia for cardiac reasons, or worsening of HF when compared to placebo in dogs with moderate to severe mitral regurgitation (MR) caused by MMVD (myxomatous mitral valve disease).

2. Materials and Methods

Animals

221 dogs were enrolled from 32 practices in France, Germany, Belgium and Italy, between February 2003 and March 2005. The study was completed in May 2006.

Study design

This multicenter study was a prospective, double-blind, placebo-controlled, randomized study. The complete clinical trial process was conducted according to Good Clinical Practice (GCP). Owner consent was obtained with the option to withdraw the dog at any time. The follow-up comprised two consecutive stages. At the first stage, dogs were recruited in 2 separate studies: one 2-month study, where Furosemide treatment was mandatory at inclusion, and one 3-month study, where Furosemide was not allowed at the time of inclusion. The second stage was a 12-month study involving dogs that had completed either of the first studies. Dogs which completed the first studies were entered into the 12-month study, where they continued to receive the same trial treatment.

Inclusion criteria

At initiation of the first stage, dogs of any breed or gender were enrolled when they presented with moderate to severe MR caused by MMVD (ISACHC class II and class III). Included dogs with dilated cardiomyopathy (DCM), were excluded from analyses (see results section). Dogs must furthermore have presented at least 3 of the following clinical signs, including at least one of cough, dyspnoea, syncope and at least one of reduced activity, reduced mobility, altered demeanor. All dogs were receiving an ACEI (angiotensin converting enzyme inhibitor). Furosemide therapy was prohibited at inclusion for the 3-month study and mandatory at inclusion for the 2-month study.
All dogs which had completed one of the initial two studies could be enrolled in the 12-month study, if agreed by the owner.

Exclusion criteria
Excluded dogs were those receiving cardiac medications other than ACEI, Furosemide, Digoxin and L-carnitine, those with acute pulmonary edema, a congenital cardiac disease or a life threatening arrhythmia, or any other diagnosed medical condition or those treated with any drugs which could interact with the assessment of the tested product efficacy (e.g. β-blockers, calcium channel inhibitors). In absence of safety data, pregnant females were not enrolled.

Randomization and blinding conditions
Double-blinding conditions were maintained throughout the two stages of follow-up for investigators, owners and study monitors. Dogs kept the same study number throughout studies and so were maintained in the same treatment group: no re-allocation was carried out.

Treatment
All dogs received conventional therapy including at least an ACEI. Furosemide was allowed after D5 in the 3-month study and from at least one day before inclusion in the 2-month study. During the follow-up, veterinarians could change the dose rate, initiate or terminate Furosemide treatment. Therapy with Digoxin and/or L-carnitine was also allowed. In addition, dogs received either Spironolactone (2 mg/kg once a day with food) or a placebo.

Evaluation schedule
In both first studies, examinations had a similar schedule. Full clinical examination, thoracic radiography, electrocardiography, echocardiography, urine and blood samples were performed on Day 0. Clinical and radiographic examinations and blood sampling were performed during the first week, at Day 28 (except radiographs), Day 56 and Day 84. In the long term study, clinical and radiographic examinations and blood sampling were performed at 3-month intervals. Echocardiographic exams were undertaken at inclusion and after 6 and 12 months.

Clinical evaluation
The protocol included assessment of the clinical variables cough, dyspnea, exercise intolerance (outside mobility, activity at home, attitude at the veterinary practice) and syncope. Lateral and dorso thoracic radiographs were used to evaluate the heart size using the Buchanan Vertebral Heart Scale\(^2\) and the presence of pulmonary edema. Standard echocardiography measurements were performed. Classification of the stage of heart failure at inclusion was assessed according to the International Small Animal Cardiac Health Council classification (ISACHC)\(^6\).

Survival evaluation
The primary end-point was cardiac related death, euthanasia due to MR, or severe worsening of MR, which was defined as the need to introduce an unauthorized cardiac therapy or to increase the dose of Furosemide over 10 mg/kg/day to prevent life threatening CHF (congestive heart failure). If the dog died spontaneously or was euthanized, the investigator specified whether the cause of death was cardiac or non-cardiac and noted the precise reason. Cardiac mortality was assessed by pooling natural deaths and euthanasia owing to cardiac causes.

Statistical analyses
All analyses were two-tailed. The descriptive analysis and initial comparability between treatment groups were made on the characteristics and clinical criteria recorded at inclusion in the two first studies. The baseline data were compared between treatment groups, to check that owner withdrawals had not produced bias, and also between these two studies (study effect).

The percentage of morbidity-mortality or mortality events at the end of follow-up was compared between groups using Fisher’s exact test. Survival curves were obtained by the Kaplan-Meier method. The survival analysis was performed by log rank test, for comparing the survival of the two treatment groups, and by a multifactorial Cox proportional hazard model to assess the impact of some covariates. The hazard ratio and its 95% confidence interval were also evaluated. Level of significance was set at p<0.05. Values were reported as mean ± standard deviation.

3. Results
Baseline characteristics of dogs at inclusion
221 dogs were enrolled. 7 dogs with DCM were excluded from further analysis, 2 dogs were lost before the first examination, which left 212 dogs with MMVD (Intention To Treat population). The baseline data were comparable between the 2 treatment groups. Because of different study protocols of the two short-term studies (regarding the use of Furosemide), there were significant differences between the studies, but those did not produce any differences between treatment groups. 123 of the 179 dogs which completed the first studies continued into the 12-month study. The remaining 56 cases (well-balanced between the 2 groups) were not enrolled because the owners were reluctant to adhere to the re-examinations in the 12-month study.

Overall outcome
Effect of therapy on outcome
In the Spironolactone and control groups respectively, 34.3% and 40% of dogs completed the 15-month period. In the Spironolactone group, 10.8% of dogs reached the primary end-point and 25.5% in the control group (Fisher’s exact test, p=0.0346). Causes of withdrawals not related to HF included the owner’s wish to stop after the first studies, concomitant disease, the owners moved away and a car accident.

The estimated 15 months survival rate was 84% for the Spironolactone dogs and 86% for the control dogs (Log rank test, p=0.017). If only mortality (cardiac death and euthanasia related to MR) is considered, the 15 months survival rate was 92% in the Spironolactone group and 79% in the control group (Log rank test, p=0.0371).

Cox Proportional Hazard models
The hazard ratio of treatment effect was 0.45 (95% confidence limits [CL] [0.22-0.90], p=0.023). This represents a 55% reduction in the risk of morbidity-mortality (severe degradation, natural death, or euthanasia related to MR). If only mortality (natural death and euthanasia related to MR) was considered the hazard ratio was 0.31 (95% CL [0.13-0.76], p=0.011) which represented a significant 69% reduction in the risk of mortality.

The study of origin (2-month study or 3-month study) and the duration of cardiac treatment before Day 0 did not affect the estimate of the hazard ratio of the treatment effect, its
confidence interval, or its statistical significance. The administration of Furosemide (well balanced in both treatment groups) significantly reduced the survival probability. It did not affect the estimate of the hazard ratio of the treatment effect.

4. Study findings

This study demonstrated that the addition of Spironolactone to conventional cardiac therapy significantly reduces the risk of cardiac morbidity and mortality in dogs with MMVD when compared to conventional therapy alone (i.e. ACEI plus Furosemide or Digoxin, if needed). A 55% reduction in the risk of mortality was found when only cardiac mortality was analyzed. Analysis of the covariate “Furosemide” in the multifactorial Cox proportional hazard model was associated with a significant negative effect although the beneficial effect of Spironolactone was not affected. One likely explanation could be that dogs receiving Furosemide had more advanced disease at study inclusion.

The baseline data and survival analyses indicate that the included population consisted of 89.8% of MMVD-cases starting the study at ISACHC class II. Hence, the estimated survival rate (morbidity-mortality) is 69% in our control group at 15 months. The rate of premature withdrawals (morbidity-mortality) is 66% in our control group at 15-months follow up, demonstrating that the benefit of Spironolactone treatment is additional to that derived from the use of ACE inhibitors alone. Considering the reduction in the risk of mortality from cardiac causes, the results in dogs were even more marked with a 69% reduction at 15 months (compared to the 31% reduction observed at 3 years in the RALE Study in human medicine). The beneficial effects of Spironolactone on survival may not only be explained by the diuretic effect of the drug. In rats and human patients aldosterone induces myocardial and perivascular fibrosis and alters the endothelial function of vessels. Studies performed in human patients with CHF showed that these effects are counteracted by aldosterone antagonists.

Recent literature demonstrated that a proportion of dogs with naturally occurring MMVD had intramyocardial arterial changes which were associated with areas of fibrosis in the myocardium, so called replacement fibrosis. The same authors suggest that more severe intramyocardial arteriosclerotic changes and more severe replacement fibrosis shorten the survival time from the onset of cardiac therapy to cardiac death or euthanasia. We believe that the observed beneficial effect of Spironolactone on survival time in the present study could partly be related to a counteractive effect of Spironolactone on the arterial changes and the replacement fibrosis.

The small size of the DCM population did not permit a dedicated analysis. The benefit of Spironolactone in this pathology should be more deeply investigated.

In conclusion, this clinical trial concerning Spironolactone therapy over a 15-month period in dogs with moderate to severe MR caused by MMVD demonstrated a beneficial effect of Spironolactone when added to conventional therapy. The risk of cardiac related death/euthanasia was reduced by 69%, as compared to conventional therapy alone (i.e., ACEI plus Furosemide or Digoxin, if needed). This finding supports Spironolactone as part of the treatment protocol in dogs with MMVD.

References


Endnotes

Martin Bland is Professor of Health Statistics in the Department of Health Sciences, University of York. He leads and teaches an M.Sc. module in Clinical Biostatistics and leads and jointly teaches a module on Measurement in Health and Disease. He also contributes sessions to M.Sc. modules in Research Methods, Biostatistics in Research Practice, and Systematic Reviews. He is involved in short course on Clinical Trials, Statistics for Clinical Trials, and Epidemiology and Statistics.

He is particularly interested in the design and analysis of studies of medical measurement and in cluster sampling in both clinical trials and observational studies. He was involved in several clinical trials in human medicine such as ICSS, a comparison of angioplasty and stenting with surgery for the treatment of occlusions of the carotid artery. He is on the editorial board of Statistical Methods in Medical Research. He is author of An Introduction to Medical Statistics and Statistical Questions in Evidence Based Medicine and together with Douglas Altman writes the Statistics Notes series in the British Medical Journal.

**PRINCIPLES OF SURVIVAL ANALYSIS ILLUSTRATED BY CEVA’S SPIRONOLACTONE TRIALS**

**Time to event data**

In health care research we often measure the time which elapses until some event occurs. We call such data time to event data. Sometimes the event is adverse, such as death, sometimes it is beneficial, such as healing. Because of the examples of time to event data which were first studied, such data are often known as survival or failure time data. The terminal event, death, healing, etc. is called the endpoint. The statistical techniques developed to deal with them are known collectively as survival analysis.

The analysis of time to event data would not require any special methods if we knew the time to event of every subject. What makes time to event data difficult to analyse is that often we do not know the exact survival times of all cases. Some subjects will still be surviving when we want to analyse the data. For some events, such as conception or readmission to hospital, the event may never happen for some subjects. Furthermore, when participants have entered the study at different times, some of the recent entrants may be surviving, but have been observed for a short time only. Their observed survival time may be less than those participants admitted early in the study and who have since experienced the event. When we know for some subjects only that the time to the event is greater than some value, we say that the data are censored. This also known as being withdrawn from follow-up.

Here we shall use the example of the CEVA Santé Animale trials of Spironolactone for the treatment of heart failure in dogs. This was a double-blind randomised trial comparing Spironolactone against placebo (the Control group). The event was death or euthanasia because of heart failure and the time to death or euthanasia was recorded in days. Censoring occurred because some animals had not died and were still alive at the time of analysis, because some dogs were withdrawn from trial at owners’ request, usually because they no longer wished to have the regular assessments of the dog, and because some dogs died from other causes, such as road traffic accidents. Data are for 15 months of follow-up.

**Kaplan Meier survival estimates**

Some censored times may be shorter than some times to events. We overcome this difficulty by the construction of what we call a life table. We follow a hypothetical cohort from any time point onwards. I shall show how this works through the example.
The beginning is unchanged. If we multiply 0.9909091 by 1.0000000 we still have 0.9909091. However, there is an effect, because the number of dogs who were observed at the start of the next day has been reduced by one. On day 8, when there was a death, there were only 108 at risk, not 109. The proportion surviving day 8 is 107/108 = 0.9907407. We get the proportion surviving from the beginning of the life table to the end of day 8 by multiplying the proportion surviving up to day 8 by the proportion surviving on day 8: 0.9909091 × 0.9907407 = 0.9817340. Now this is not the same as the proportion known to have survived beyond day 8 from the beginning, which would be 107/110 = 0.9727272. We have adjusted the estimate for the dog who was censored on day 7. This dog contributed information for the time in which it was observed and we have included this information in the analysis.

Table 1 shows the survival times for the Control group. Each time is marked ‘D’ for a heart failure death at that time and ‘C’ if the data were censored. Thus, in the first column, the first time was 6 days and the dog died. The second time was 7 days and the dog was censored.

The start of the calculation is set out in Table 2. For each time when an event or censoring occurs, we find the number of dogs who were present at that time, called the number at risk. There were 110 dogs at the start. We find the number who died. For the first time when an event took place, 6 days, there was one death, leaving 110 – 1 = 109 dogs who did not die. This is the number surviving to the end of day 6. We then calculate the proportion who survived to the next time. At 6 days, this is 109/110 = 0.9909091. This is the proportion of those starting day 6 who survived to the end of day 6. On day 7 there is a censoring, but no deaths. The proportion of dogs surviving is 1.0000000, and the total survival from the beginning is unchanged. If we multiply 0.9909091 by 1.0000000 we still have 0.9909091. However, there is an effect, because the number of dogs who were observed at the start of the next day has been reduced by one. On day 8, when there was a death, there were only 108 at risk, not 109. The proportion surviving day 8 is 107/108 = 0.9907407. We get the proportion surviving from the beginning of the life table to the end of day 8 by multiplying the proportion surviving up to day 8 by the proportion surviving on day 8: 0.9909091 × 0.9907407 = 0.9817340. Now this is not the same as the proportion known to have survived beyond day 8 from the beginning, which would be 107/110 = 0.9727272. We have adjusted the estimate for the dog who was censored on day 7. This dog contributed information for the time in which it was observed and we have included this information in the analysis.

Table 2. Calculation of the Kaplan Meier survival estimates for the Control group (beginning only)

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The survival estimate is only an estimate, and if we were to repeat the trial with a new sample of dogs we would get a different survival curve. To allow for this uncertainty, we can add a 95% confidence interval for the survival estimate. This gives what are called Greenwood bounds around the survival curve. This gives a range within which we estimate the survival for all dogs in the population to be. Figure 3 shows these for the Control group.

We can compare the two arms of the trial, as shown in Figure 2. We can see that Spironolactone appears to have better survival, because the curve is above that for the Control group.
The survival curve shows the estimated proportion surviving at any chosen time, but we can also estimate the time to which any chosen proportion of dogs will survive. For example, we might want to find the median survival time, the time to which half the dogs survive. We draw a horizontal line through the chosen proportion, 0.50, to intersect the survival curve (Figure 4).

However, the survival curve and the horizontal line do not meet. We cannot estimate the median survival time, because too few dogs have died at the time of follow-up. We cannot estimate the median survival time, because too few dogs have died at the time of follow-up. We cannot estimate the median survival time, because too few dogs have died at the time of follow-up.

Figure 4. Attempt to estimate the median survival time for the Control group

But it is not a precise estimate. The lower limit of the 95% confidence interval is 154 days. However, the upper limit does not exist. The 0.75 survival line does not cross the upper Greenwood bound. Too few dogs have died for us to produce a meaningful estimate.

We can find the 75% survival time, when 25% of the dogs have died, as shown in Figure 5. We draw vertically down to the time axis to estimate the 75% survival time as 335 days.

Figure 5. Estimation of the 75% survival time for the Control group

We can find the 95% confidence interval for this estimate by drawing downwards from where our 75% line intersects the Greenwood bounds, as shown in Figure 6.

Figure 6. Confidence interval for the 75% survival time for the Control group

The logrank test

We want to be able to compare the survival in the treatment groups, to say whether there is good evidence for a real difference between the treatments (that is, whether the difference is statistically significant) and to estimate how big that difference is. Greenwood standard errors and confidence intervals for the survival probabilities are useful for estimates such as the one year survival rate. They are not a good method for comparing survival curves. They do not include all the data and the comparison would depend on the time chosen.

Instead of the Greenwood method, to compare survival curves we need a method which makes use of the full survival data. There are several significance tests which we can use for this, of which the best known is the logrank test. This is a non-parametric test which makes use of the full survival data without making any assumption about the shape of the survival curve.

The logrank test tests the null hypothesis that, at any time, the chance of a member of a group experiencing an event is the same for both groups, though the actual chance of an event may change over time. The alternative hypothesis is that at some time the chance of an event is different in the two groups, which would make the survival curves different.

We calculate how many of the 28 deaths observed would be expected to be in the Spironolactone group and how many would be expected in the control group, if there were no difference between Spironolactone and placebo. The precise number expected depends on the pattern of censoring in the groups. On each day where there are deaths, for each group we calculate the total number of deaths for the day divided by the number in the group. Then we split the number of deaths between the two groups in proportion to the numbers at risk. We then add these for each group. For the trial data, we would expect 13.11 deaths in the Spironolactone group and 14.89 deaths in the Control group. We actually observed 6 deaths in the Spironolactone group and 22 deaths in the Control group. The probability of a difference this large between what we observe and what we would expect is 0.007. The difference is statistically highly significant.

The hazard ratio

To produce an estimate of the size of the difference in survival, we have to make some assumptions about the shape of the curve. We have to assume that they are similar in some way, so that we can find some numerical value to compare between them. We can use the Greenwood standard errors to find a confidence interval for the difference between the survival probabilities at a given time, but this does not use all the data, events after the chosen time being ignored.

The best way to estimate the difference between the survival curves uses the hazard, which is a measure of the chance that a member of the population will have an event at any given time,
or the rate at which events happen. To be more precise, we find the probability of an event in any small time interval by multiplying the width of the time interval by the hazard at that time. Hazard depends on the survival time, so that it might increase or decrease as follow-up goes on. If we can assume that the survival curves in the two treatment groups follow the same pattern, then we can assume that if hazard is greater in one group than in the other at one time, it will also be greater at another time. If this is the case, we assume that the hazard in one group is equal to the hazard in the other group multiplied by a constant number, which we will estimate. Thus, if members of one group have twice the risk of an event for members of the other group on the first day, they will also have twice the risk of the event on the second day, twice the risk on the third day, and so on.

The constant ratio is called the hazard ratio. If the risk of an event is the same in the two groups, the hazard ratio is equal to one. If the risk is lower in the intervention group than in the control group, the hazard ratio is less than one. If the risk is greater in the intervention group, the hazard ratio is greater than one. For the hazard in the Spironolactone group divided by the hazard in the Control group, the hazard ratio is 0.31 with 95% confidence interval 0.13 to 0.76. At any given time, the risk of death in the Spironolactone group is estimated to be one third of that in the Control group.

We can adjust the treatment hazard ratio for other variables which may influence survival, using a method called Cox proportional hazards regression. One obvious variable to adjust for is study of origin, the dogs having originally been in two different medium-term studies. These were then combined to produce the long-term study. The adjustment has no discernable effect, the hazard ratio and confidence interval being unchanged to two decimal places.

Other variables were used in the earlier analysis as potential predictors of survival: the duration of cardiac therapy before inclusion, and concomitant treatment with furosemide. In Cox regression, we usually limit the number of variables to at most one per 10 events. We have 28 deaths among the dogs, so here the maximum number of predictors for a reliable analysis is three. We can add each of these variables to the regression on treatment and original study, separately. When we do this, there is virtually no effect on survival and the hazard ratio for treatment with Spironolactone remains almost unchanged.

Presenting the results of survival analysis

The hazard ratio is the usual statistic used to report the results of survival analysis in the medical literature, where such analyses are much more common than in the veterinary literature. For example, we can see this in major medical journals. The Lancet is one of the most highly cited international medical journals and reports many studies using survival data. In the first three months of 2007, there were 13 papers which reported the results of survival data in the Summary. Of these 13 papers, 10 reported randomised trials. The main hazard ratios for these trials were: 0.40, 0.47, 0.64, 0.86, 0.89, 0.76, 0.78, 0.81, 0.83, 0.97. (Where hazard ratios were greater than 1.0, I have reversed the order of treatments to make them comparable to the Spironolactone trial). All of these were closer to 1.0 (no effect) than the 0.31 observed for Spironolactone and the mean hazard ratio = 0.70. Hence this trial has produced an unusually large effect.

Survival analysis has proved so valuable that Kaplan and Meier (1958) and Cox (1972) are the two mostly highly cited statistical papers to date (Ryan and Woodall, 2005).

References


**ANSWERS FROM:**

**CLAUDIO BUSSADORI (CB)**  
DVM, MD, DipECEHM, PhD – Director, Clinica Veterina Gran Sasso and Researcher, Paediatric Cardiology Department, S. Donato Hospital, Milan, Italy

**JOHN MARTIN BLAND (JMB)**  
PhD, MSc, DIC, ARCS, FSS, Hon. MRCR – Professor of Health Statistics, Department of Health Statistics, University of York, York, UK

**LAURE BADUEL (LB)**  
DVM, Head of the Pre-clinical and Clinical Department, Ceva Santé Animale

**Question (Delegate) - How can we keep confidence in trial, such as this, where there is a high censor rate?**  
**Answer (UMB) -** Many censorings were because owners did not wish the dog to continue with the trial. The proportion of dogs which were withdrawn at the end of the short-term studies or which died was very similar in the two groups. This is a double blind trial and neither owners nor vets knew which treatment the dog was receiving, so they did not withdraw because they were not happy with the treatment to which the dog had been allocated. Survival curves were drawn considering censored data. The efficacy of spironolactone was demonstrated comparing these two curves.

**Comment (Speaker: Faiez Zannad, France) -** A mortality trial is not designed to identify how a treatment works. One way of looking at whether spironolactone had a diuretic effect would be to compare the doses of frusemide in the treatment and control groups. If there was a diuretic effect of spironolactone, then one would expect a reduced dose of frusemide in the treated dogs.

**Answer (LB) -** We attempted to follow the frusemide doses and found no differences between the two groups in mean dosages used. But we were limited in this assessment because of clinical treatment reasons, for example whether the frusemide was given by intravenous or oral routes.

**Question (Speaker: Nicolette Farman, France) -** Is there a difference between the responses of male and female dogs to spironolactone?

**Answer (LB) -** We observed no difference between sexes in response.

**Answer (CB) -** We must remember that it is male dogs that are more frequently affected by myxomatous mitral valve disease.

**Question (Speaker: Johann Bauersachs, Germany) -** First, have you had a chance to look at the echocardiographic data? Second, did you prevent the development of hypokalaemia at the dose of spironolactone used?

**Answer (CB) -** We haven’t yet looked at the echocardiography data, largely for the reason that there is too much variability in the methods used to measure different echocardiographic parameters by clinicians across Europe. This is always a difficulty in this type of study. As for your second question, hypokalaemia is rare in my experience and limited to a few cases that develop trembles and a loose neck carriage. These are usually seen when the dose of furosemide is >5mg/kg twice daily. I haven’t seen problems when combining ACE inhibitor and furosemide.

**Question (Delegate) -** The number of cases in the clinical trial were in ISACHC (International Small Animal Cardiac Health Council) stage III (advanced heart failure) was very low. Should you be able to exclude these and look specifically at those in stage II (mild to moderate heart failure)?

**Answer (LB) -** We haven’t done that; the trial wasn’t designed to do that.

**Question (Delegate) -** How do you prove the diuretic effect of spironolactone? Is there any evidence of weight loss or oedema reduction?

**Answer (CB) -** There was no direct evidence of the diuretic effect, although we were not looking specifically at this.

**Answer (LB) -** We sampled urine, but only collected a single sample every 1-3 months and this wasn’t always at the same time each day. It is therefore very difficult to interpret the results and assess the diuretic effect. It is not like in experimental conditions where you can collect urine over a 24 hour period.

**Answer (CB) -** In reality it is impossible to differentiate in this study between the effects of frusemide and spironolactone on diuresis.

**Question (Chair) -** In veterinary medicine, where there is the option for euthanasia, how do you best account for this end point? Especially given that different owners may differ in their preference or decision making.

**Answer (UMB) -** The answer is simple. Do a double-blind, placebo-control study, then nobody knows the treatment and cannot alter the decision to euthanize.

**Question -** Is there any validity in censoring these data?

**Answer (UMB) -** You should definitely not censor these data. You should only do that where deaths are due to other causes, e.g. road traffic accidents.

**Question (Delegate) -** How do you prove the diuretic effect of spironolactone? Is there any evidence of weight loss or oedema reduction?

**Answer (CB) -** There was no direct evidence of the diuretic effect, although we were not looking specifically at this.

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**Answer (CB) -** In reality it is impossible to differentiate in this study between the effects of frusemide and spironolactone on diuresis.
Question (Chair) - Just to follow up Dr Schneider’s point, Claudio indicated that the dogs were in mild to moderate Mitral Valve Disease. Did you exclude those with pulmonary oedema on radiographs?
Answer (CB) - Yes, those with acute pulmonary oedema on thoracic radiographs were excluded.

Question (Chair) - Did any of the dogs have primary respiratory disease as the cause of their cough?
Answer (CB) - We did chest films in all cases in an effort to exclude these cases.
Answer (LB) - If we did miss them, then there is every likelihood that such cases would be balanced between the spironolactone and control groups.

Question (Delegate) - I have a comment as to the potassium status. People, dogs and cats are all different. It is amazing how tolerant dogs are to hypokalaemia.
Answer (CB) - I agree, dogs are very different in this respect.

Question (Speaker: Robert Hamlin, USA) - Does the EMEA (European Medicines Agency) draw a distinction between clinically and statistically significant differences?
Answer (JMB) - I can’t respond on behalf of the EMEA but I would say that, just because something is statistically significant that doesn’t make it clinically important. The evidence from this trial is that spironolactone is associated with better survival, but how much better?

Comment (Chair) - John Rush and colleagues at Tufts did a study looking at what owners value in terms of time and quality of life with their pets. The majority selected a period of 6 months but would be happy with an additional 3-6 months.
Answer (CB) - It is difficult to understand what quality of life means. Owners accept very different outcomes; it is a subjective matter and difficult to predict the views of individual owners.
Answer (JMB) - I would only add that when Richard Peto launched his call for large but simple clinical trials, he was only looking for a 10% reduction in mortality as a measure of significance.
Faiez Zannad is Professor of Therapeutics and Cardiology. He is at the Head of the Division of Heart Failure, Hypertension and Preventive Cardiology for the department of Cardiovascular Disease of the Academic Hospital (CHU) in Nancy and the Director of the Clinical Investigation Centre (INSERM-CHU) of Nancy since 1995. He entered the European Society of Cardiology (ESC) in 1996 and is currently the Chairman of the ESC Working group on Pharmacology and Drug Therapy as well as a Board member of the ESC Heart Failure Association. He is Past-President of the French Society of Hypertension.

As the Coordinator of French Cardiovascular Clinical Investigation Centres, he has participated in various famous large scale trials in human cardiology such as RALES, VALIANT, CIBIS, CAPRICORN or EPHESUS. In these trials, he has been involved either as a member of the Steering Committees or in the Protocol Writing Groups. He is Co-Editor-in-Chief for Fundamental and Clinical Pharmacology, the official journal of the European Pharmacology Societies Federation (EUPHAR). He chairs and organises annual international meetings on Cardiovascular Clinical Trials (CVCT) and on Biomarkers in Heart Failure.

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**CURRENT CLINICAL STATUS AND FUTURE DIRECTIONS FOR BIOMARKERS IN HEART FAILURE**

**Introduction:**

Biomarkers provide the clinical cardiologist with a number of laboratory tests for defining the molecular diagnosis, assessing new risk factors, and better targeting the pharmaceutical approaches in patients with cardiovascular disease. An increasing number of novel risk factors have been added to the classical risk factors of cardiovascular disease. The recent surge of genetic analysis procedures will likely soon.

This review is focused on the current clinical status and future directions for biomarkers in heart failure. All major biomarkers currently used in clinical practice, and other candidate biomarkers are listed in Table 1, from an excellent overview 1.

**Overview of Current Knowledge**

**Natriuretic Peptides**

Three peptides comprise the natriuretic peptide family, including atrial natriuretic peptide (ANP), BNP, and C-type natriuretic peptide. Elevations of these peptides have been associated with varying degrees of cardiac impairment. Two forms of natriuretic peptide, BNP (B-Type Natriuretic Peptide) and its precursor, NT-Pro BNP, have been studied as aids to establish the diagnosis, estimate prognosis and monitor the response to therapy of patients with ADHF 2. Recent studies have established their diagnostic value in patients presenting with dyspnea, their association with prognosis and early results suggest their potential role in assessing response to therapy in ADHF. Natriuretic peptides are attractive biomarkers in HF for several reasons. An ideal biomarker should possess a high degree of sensitivity and specificity for the disease state which has been demonstrated for natriuretic peptides in HF. Their enhanced gene expression is evident in early HF, and plasma levels increase as HF progresses 3. Natriuretic peptides are closely linked to the pathophysiology of HF, as their actions include diuretic, natriuretic, vasodilatory, and anti-fibrotic effects, and they suppress renin angiotensin aldosterone system activity. The biologic responsiveness to these physiologic actions is blunted in patients with HF.

Multiple hypotheses have been proposed to explain this observed resistance to the biologic effects of natriuretic peptides 4. Numerous clinical studies described below point to their clinical utility in HF.

**Diagnostic Utility**

The diagnostic value of BNP was evaluated in the Breathing Not Properly study 5. BNP levels were significantly higher in patients with dyspnea due to congestive heart failure. McCullough et al reported the diagnostic accuracy for physician clinical judgment, BNP, and both 6. The diagnostic accuracy associated with BNP levels > 100 pg/mL was 81%,
compared with 74% for clinical judgment alone. Combining BNP with clinical judgment resulted in an area under the ROC curve of 0.93 \(^6\). In the PRIDE study \(^7\), NT-Pro BNP results were correlated with a clinical diagnosis of acute HF: NT-Pro BNP levels increase with age so that the study investigators recommended cut points of > 450 pg/ml for patients younger than 50 years of age and > 900 pg/ml for patients age 50 years or older. These cut points were associated with highly sensitive and specific for HF in this study.

**Prognostic Utility**

Serial measurements of BNP appear to provide prognostic information beyond that achieved with a single baseline determination \(^8\). Patients whose BNP increased from baseline to 4 months had a higher mortality risk as compared to patients whose BNP levels remain below the median throughout the study. Increasing BNP was also associated with increases in left ventricular end-diastolic diameter, whereas decreasing BNP was associated with decreases in this measure \(^9\). These data suggest that monitoring BNP change during follow-up may aid in patient risk stratification.

**Challenges Facing Natriuretic Peptides as Biomarkers**

While natriuretic peptides are well validated as biomarkers in HF, several cautions related to the clinical use should be recognized. Consistency in the method used to serially monitor BNP is important. Assay results for BNP may vary widely due to differences in antibody cross-reactivity \(^9\) to 10. In addition, demographics and comorbidities such as female gender, renal impairment, obesity, and atrial fibrillation may significantly influence natriuretic peptide values independent of the degree of HF present. Whereas BNP is an established indicator of decompensated HF, substantial variation in BNP levels have been reported for ambulatory patients with chronic heart failure \(^11\). As discussed in detail below, several recent reports question whether assays used in clinical practice are actually measuring the biologically active form of natriuretic peptides \(^12\) to 15. These findings indicate that altered forms of BNP contribute to the values detected by various clinical assays. Whether more specific measurement of the active form of BNP would be of greater clinical value than existing assays, remains to be determined. Other natriuretic peptide gene products could be useful HF biomarkers, but more research is needed to better identify them and determine their diagnostic and prognostic significance.

**Necrosis Markers**

Cardiac troponins are well established as the markers of choice for myocardial injury and necrosis in acute coronary syndromes. They appear in the plasma rapidly after myocardial injury with a high sensitivity and specificity \(^16\). Cardiac troponins are detectable even when creatine kinases are not, and the troponin T and I isoforms are unique for cardiac tissue. Recent studies have also documented increased circulating troponin in HF patients in the absence of acute ischemia, leading to extensive investigation of this molecule as a biomarker in this syndrome.

**Association of Troponin with Clinical Outcome**

Although elevations are modest compare to those seen in acute coronary syndrome, several studies have demonstrated an association between abnormal troponin levels and subsequent clinical events in HF \(^17\) to 19. Serial measurements of troponin may also have important value. Troponin release during follow-up is associated with a higher mortality risk and a higher risk of heart failure hospitalization. Abnormal troponin release is an attractive biomarker for HF because it seems to reflect loss and/or progressive dysfunction of cardiac myocytes. Troponin may be useful in the selection of patients who may be appropriate candidates for therapies targeting the prevention of myocardial necrosis. Troponins may also be valuable as components of a multimarker strategy to identify high risk patients. Although troponin release seems to reliably identify myocyte injury, it does not indicate a specific mechanism of injury. Further research should provide a better understanding of the mechanism(s) of troponin release and may lead to the identification of other clinically important markers of the underlying pathophysiological process of cardiac myocyte compromise and loss. Elevated troponin is a consistent predictor of mortality in HF. However, additional studies are needed to determine troponin suitability as surrogate marker of response to drug therapy.

**Fibrosis Markers**

The extracellular cardiac matrix (ECMM) plays an important role in the support of myocytes and fibroblasts. Collagen is the principal structural protein and collagen types I and III are the most abundant in the myocardium. Collagen type I has a poor specificity but represents the majority of cardiac collagen (85%) and confers tensile strength and resistance to stretch and deformation. Type III is less abundant but more specific to the heart and confers resilience \(^1\) to 3. Fibriilar collagens within the myocardium are substrates for matrix metalloproteinases (MMPs). Among the MMPs, MMP-1 has the highest affinity for fibrillar collagen and preferentially degrades collagen I and III. The net level of MMP-1 activity is dependent on the relative concentrations of active enzyme and of a family of tissue inhibitors of metalloproteinases (TIMP). MMP-1 and TIMP-1 are co-expressed in cardiac fibroblasts and are tightly regulated to maintain the architecture of the ECMM. CITP is a pyridinoline-cross-linked telopeptide produced as a result of the hydrolysis of collagen type I fibrils by MMP-1 and is a marker of collagen type I degradation. The disruption of the equilibrium between the synthesis and degradation of the ECMM results in an excessive accumulation of collagen type I and III fibers within the myocardium. ECMM remodeling is an essential process in cardiac remodeling, hypertensive cardiac hypertrophy, dilated cardiomyopathy, and post infarction healing. ECMM turnover is influenced by ischemia, stretch, inflammation, and neurohumoral mediators. Myocardial fibrosis is therefore the consequence of a number of pathologic processes mediated by mechanical, neurohumoral, and cytokine factors. Cardiac fibrosis, a major determinant of diastolic dysfunction and pumping capacity, results in tissue heterogeneity and anisotropy, provides the structural substrate for dys-synchrony and arrhythmogeneity, thus potentially contributing to the progression of congestive heart failure (HF) and sudden cardiac death. ECMM turnover may be the target of therapeutic agents aimed at preventing or limiting the progression of adverse cardiac remodeling in HF and therefore hospitalization for HF as well as death due to progressive HF and sudden cardiac death. Given the importance of fibrous tissue in the pathophysiology of myocardial dysfunction and failure the non-invasive assessment of fibrosis could prove to be a clinically useful tool, particularly given the potential for cardioprotective and cardioreparative pharmacological strategies.
The measurement of various serum peptides arising from the metabolism of collagen types I and III may provide information on the extent of myocardial fibrosis and thus prognosis as well as clues to appropriate strategies to improve prognosis. Since procollagen type I C-terminal propeptide (PICP), aminoterminal propeptides of type-I procollagen (PINP), and N terminal type III collagen peptide (PIIINP) are released with collagen type I or III molecules in a stoichiometric manner during collagen biosynthesis, they are important markers of this process. Although these markers are not specific to the myocardium, studies have shown a correlation between myocardial collagen content and the serum concentration of PICP in patients with hypertension and have demonstrated that serum PICP is secreted by the heart via the coronary sinus in patients with hypertensive heart disease. The PIC/PITP ratio, an index of coupling between the synthesis and degradation of collagen type I, was found to be higher in hypertensive patients with increased collagen accumulation in myocardial tissue than in those with normal collagen accumulation. This evidence linking serum ECCM markers to the heart ECCM content provides a rationale for their use as biomarkers of ECCM remodeling in cardiac disease. MMP-1 and TIMP-1 levels in coronary sinus blood are higher than in peripheral venous blood in hypertensive patients, but not in normotensive subjects, although there was no association between blood levels and myocardial expression of MMP-1 and TIMP-1 or the amount and distribution of fibrillar collagen. Biomarkers reflecting collagen formation and/or degradation may be used for early detection of otherwise sub clinical disease; diagnostic assessment of acute or chronic clinical syndromes; risk stratification of patients with confirmed disease; selection of appropriate therapeutic interventions; and monitoring the response to these interventions. ECCM biomarkers in patients with HF may detect early changes in heart and large vessel structure and function, the transition to heart failure, and prognosis. The ability of treatment to reduce myocardial fibrosis in patients with HF may be monitored by the measurement of various serum peptides arising from the metabolism of collagen types. Characterization of patients according to the severity of cardiac fibrosis, as assessed by ECCM biomarkers, may prove useful for selecting appropriate drug regimens, such as aldosterone antagonists. The available data set the stage for large scale long-term randomized trials to validate this approach. However, before wide spread application of this approach it will be necessary, as pointed out above, to standardize the various measurements of ECCM biomarkers and to recognize their limitations. In part these limitations may be overcome by the concomitant use of new imaging techniques to localize myocardial fibrosis such as radionuclide angiography with labeled intergrins as well as magnetic resonance imaging.

**Multimarker Strategy**

Since several biomarkers appear informative for the diagnosis and prognosis of HF, interest has developed in the potential clinical use of multiple markers simultaneously in individual patients. Combining biomarkers incorporates potentially additive information and may provide a more accurate representation of a patient’s risk profile. Ishi et al studied 98 patients hospitalized for decompensated HF. Troponin T, troponin I, and BNP were obtained at the time of admission. The optimal value of troponin and BNP to predict clinical events was determined by a receiver operating curve approach. Patients were categorized into low, intermediate, and high risk groups according to troponin and BNP levels. As expected, low risk patients had low troponin and low BNP. Intermediate risk patients had either troponin or BNP above the optimal prediction level, while high risk patients had both troponin and BNP above the optimal prediction level. The combination of troponin and BNP provided a more accurate risk stratification than either of the parameters alone. The authors of this study replicated their findings in a separate patient cohort. Similar findings have also been reported by Horwich et al. Patients with elevations of both BNP and troponin had the highest mortality in a cohort of 238 patients with advanced HF undergoing transplant evaluation. Multimarker strategies are attractive because they allow the integration of several components of the pathophysiologic process. Combining BNP and troponin values incorporates an assessment of both congestion and myocardial injury. HF is a complex syndrome with multiple pathophysiologic processes that occur simultaneously. Adopting a multimarker strategy may yield more accurate diagnostic and prognostic information since it incorporates more than one aspect of the disease process.

**Novel Potential Biomarkers in Heart Failure**

**(Table 1)**

**Cytokines: Interleukin 1B**

Changes in the elaboration of many cytokines have been identified in HF but no specific cytokine has emerged as a clinically useful biomarker to date. The search for novel cytokines as biomarkers in HF continues. Abnormally elevated levels of interleukin-1B (IL-1B) have recently been detected in a small study of patients with heart failure, and ANP mRNA expression increased in IL-1B treated myocytes. In addition, IL-1B levels have been associated with symptom severity. In a study of 66 patients with NYHA Class II-IV heart failure, IL-1B levels were significantly higher among patients with NYHA Class IV symptoms as compared to NYHA Class II or III. Another study reported higher levels of IL-1B in patients with HF as compared to normal controls, and IL-1B was also associated with NYHA Class. In contrast in this patient population, IL-6 was not associated with functional class, and there was no detectable correlation between IL-6 and IL-1B. White et al recently reported the results of a study in 29 patients with worsening HF who received 72 hours of milrinone or dobutamine therapy. IL-1B was significantly higher as compared to age matched controls on admission while at 30 days, a significant reduction in IL-1B and other markers of inflammation was observed. These studies suggest the potential for IL-1B as a biomarker in HF but additional studies will be required to determine if this cytokine will be of clinical value in predicting outcome or monitoring therapy in HF.

**Other Natriuretic Peptides**

The family of natriuretic peptides consists of several members, but most clinical recommendations focus only on the assessment of BNP and its precursor NT-Pro BNP. Currently, both are considered superior to atrial natriuretic peptide (ANP) for diagnostic and prognostic assessment of patients with HF, even though ANP comprises most of the natriuretic peptides in the circulation. The assessment of ANP is less reproducible, but measurement of ANP’s precursor proANP may overcome the problem of reproducibility, since it...
is significantly more stable in the circulation than the mature peptide. Assessment of other natriuretic peptides may prove valuable. The predictive value of mid-regional proANP, a novel natriuretic peptide was assessed in a cohort of 525 patients with stable HF. Mid-regional proANP was as powerful as NT-ProBNP in predicting mortality in these patients. Mid-regional proANP was superior to NT-ProBNP in important subgroups of patients in which NT-ProBNP failed to predict survival. This was particularly evident in patients with mild disease or the obese. Additional studies in other patient populations with HF are needed to fully validate this marker, but these studies provide support for the need to monitor multiple markers. Other natriuretic biomarkers such as mid-regional pro-adrenomedullin may also prove to be useful.

Novel Biomedomic Approaches

Recent advances in molecular biology methods promise to transform the current approach to detection and application of biomarkers in heart failure. Specific examples of recent data from these rapidly evolving fields (based on proteomic and transcriptomic methods) illustrate their potential to fundamentally alter our approach to biomarker characterization of HF.

Potential for biomarkers to guide therapy

Conceptually, the use of biomarkers to guide therapeutic decisions is attractive and already supported by a variety of observational studies and a few preliminary clinical trials. However, no biomarker to date has been clearly established for this indication in HF. Interest continues to be driven by the lack of precision in early clinical diagnosis and lack of reliable indicators to guide the therapy in established HF. Many HF patients remain at high risk despite advances in therapy. Using classical clinical assessments, there has been a significant increase in treatment gaps in the use of evidence-based medication which persist despite strong positive results from randomized clinical trials. Observational studies have demonstrated that biomarker measurements provide a more accurate assessment of risk than clinical assessment alone and suggest that knowledge of biomarkers could help optimize therapy in HF. As a foundation and rationale for larger outcomes studies, preliminary findings are now available from several recent pilot trials on biomarker guided therapy in HF. These results include findings from the Strategies for Tailoring Advanced Heart Failure Regimens in the Outpatient Setting: Brain Natriuretic Peptide Versus the Clinical Congestion Score (STARBRUTE) trial and STARS (Treatment monitoring of systolic cardiac insufficiency), which were designed to evaluate whether a BNP guided approach to managing fluid balance is more effective at preventing death or hospitalization as compared to clinical assessment alone in patients hospitalized with acute decompensated HF. These studies provide substantial support for large-scale outcomes studies of the efficacy of monitoring therapy based on BNP. Troponin is another candidate for consideration as a marker to guide management of patients with heart failure. Although the mechanisms responsible for troponin release are not well understood, elevated troponin detected by current assays does appear to reflect true cardiac myocyte loss and has been strongly associated with adverse outcomes. These findings suggest troponin could serve as a guide/surrogate marker of therapeutic response in HF patients. The proposed rational concept assumes that a therapeutic regimen associated with increased troponin may have a negative effect on clinical outcomes, whereas treatments associated with normalization of troponin or no troponin release is likely to be associated with favorable outcomes. Whether troponin release is secondary to ischemia, remodeling, or elevated filling pressures, targeting these conditions with adjustments of various therapeutic interventions may result in reduced myocyte loss and improved outcomes.

Despite early favorable data, there are still several important aspects of biomarker guided therapy to carefully consider in testing this strategy: 1) benefit and risk associated with specific treatment adjustments in response to changes in biomarkers; 2) use of single versus multiple markers to guide therapy; 3) extent of change in biomarker(s) necessary to alter therapy; and 4) target HF patient population for guided therapy.

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References


ANSWERS FROM:

FAIEZ ZANNAD
MD, PhD – Professor of Therapeutics and Cardiology, Department of Cardiology, CHU and University Henri Poincaré, Nancy, France

Question (Delegate) - First, what is the physiological basis for matrix metalloproteinases (MMP) as cardiac biomarkers? Second, in which diseases have MMP been studied. Third, would you see changes in MMP before end stage heart failure?

Answer - The questions relate to what extent the markers reflect collagen synthesis versus degradation, and whether it is an acute or chronic disease state. MMPs are involved in collagen degradation and are much more complex to measure than the other collagen biomarkers, suffer from high variability and are unstable to store. This has limited their use.

Question (Delegate) - In order to circumvent daily variation within BNP and other biomarkers, are there systems such as fructosamine that look at longer term values.

Answer - Biomarker companies are actively looking for more stable fragment, so-called memory markers but these have not yet reached the clinical setting.

Question (Chair) - Would that include tests for proBNP?

Answer - It is a matter of where you are, since there are regional preferences for the way in which tests are used.

Question (Chair) - Are there actually assays for the prohormone?

Answer - Yes, but it is unclear whether they measure the prohormone or specific fragments, and we await more data.

Question (Delegate) - If and how much does inter-daily and inter-weekly individual variability affect cardiac biomarkers?

Answer - Synthesis biomarkers, like PIIINP are extremely robust. BNP however, is much more variable from one day to another.

Question (Chair) - Do you know if a patients water intake or the amount of salt they had in their diet or time of day influence what their value might be when they come into the hospital?

Answer - BNP has a very short turnover so it is very much related to congestion and hemodynamic conditions. Collagen biomarkers are more stable, just because the turnover and the half-life are extremely long. 120 days is the half-life of collagen renewal in the heart. There are short-term variations in acute myocardial infarction or acute heart failure, for example, but in chronic conditions, it is rather stable.

Question (Delegate) - There is an elevation of BNP in late heart failure. Is this due to resistance to the peptide?

Answer - Ageing is important. BNP is very useful in acute heart failure trials as it indicates congestion. But there is also a kind of tachyphylaxis in those patients with heart failure because they have high level of BNP and so they are more resistant to BNP infusions.

Question (Chair) - If and how much does inter-daily and inter-weekly individual variability affect cardiac biomarkers?
Mark Oyama is an Associate Professor in the Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania. His main clinical and research interests involve canine myocardial disease, mitral valve disease and cardiac biomarkers. He is currently involved in several research projects dealing with investigation of biomarkers in dogs and cats as well as studies investigating serotonin-related molecular mechanisms of canine valve disease.

He is the current President of the American College of Veterinary Internal Medicine, Specialty of Cardiology and a member of the ACVIM Board of Regents. He has served as a member of the NIH-Center for Scientific Review Bioengineering, Technology, and Surgical Sciences study section and is a member of the University of Pennsylvania Institute of Translational Medicine and Therapeutics. Dr. Oyama has published over 90 scientific manuscripts and abstracts and has given over 150 national and international lectures. He serves on veterinary advisory boards for a variety of biotechnology, pharmaceutical, and diagnostic laboratory companies and is the Translational Sciences section editor for the Journal of Veterinary Cardiology. He resides in Philadelphia with a Golden retriever and two very mischievous cats.

Jonathan Elliott graduated from Cambridge Veterinary School in 1985. After a year as an Intern at the University of Pennsylvania, he undertook a PhD in vascular pharmacology in Cambridge.

In 1993 he was appointed to a lecturership in Veterinary Pharmacology at the Royal Veterinary College and developed research interests in feline kidney disease and hypertension and Equine Laminitis. He was awarded the Pfizer Academic Award in 1998 and the BSAVA Amoroso Award in 2001, the Petplan Scientific Award in 2005 and the ESVNU Award in 2007 for contributions to companion animal medicine. He was a member of the ACVIM Consensus Statement Panels on Proteinuria and Hypertension and chaired the International Renal Interest Society from 2002-2004.

He is currently Professor in Veterinary Clinical Pharmacology and Vice Principal for Research at the RVC and is a Diplomate of the European College of Pharmacology and Toxicology and a member of the UK Government’s Veterinary Products Committee.
CEVA Santé Animale is the world’s largest animal health company, researching, developing, producing, and marketing pharmaceutical products and vaccines for companion animals, livestock, and poultry. The company is based in Libourne, France and is directly invested in 45 other countries with distributive partners in many more, giving us a truly global dimension.

A results culture:

- Originally a subsidiary of the Sanofi group, the Sanofi Santé Nutrition Animale (SSNA) management conducted the first of three leveraged buyouts to launch in 1999 CEVA Santé Animale.
- In the latest LBO completed during 2007, the CEVA Santé Animale’s management and employees assumed a majority stake in the company with the backing of financial partners Euromezzanine and Natixis.

During our first 10 years of existence, the company’s organic growth has averaged 8.4%, well beyond the industry average. We owe our existence to the farmers, pet owners, and veterinarians who support us and as a result we are totally dedicated to providing them with products and services that protect and improve not only the lives of their animals but the well-being of us all, as a global community.

Key figures (Dec 2008):
- Sales: Euro 363m.
- Operating income: 49.4m.
- R&D expenditure: 27.6m (7.7% sales)
- Employees: 2175

Innovation is a frequently used word in the pharmaceutical world often associated with the introduction of new “blockbuster” products. CEVA’s approach to innovation, aside from our R&D programme, is to encourage our local and central teams to find better solutions to existing and emerging diseases, which will in turn improve animal health and productivity.

That’s what we mean when we use the word GLOCAL: our aim is to be global in our ambition to tackle the big issues and local in the way that we tailor practical solutions for our customers.

The bigger picture: 75% of emerging human diseases are of animal origin (source: OIE)

CEVA is heavily involved in the fight to protect against diseases such as avian influenza and brucellosis that also directly menace the human population. Our strength and commitment in addressing these diseases goes far beyond our relative market position, this is equally true of our commitment to the emerging markets of the world, where we have invested significantly despite the level of risk.

We believe that a “one health, one planet” approach is required to properly address global animal health issues and we will continue to invest our time and resource to ensure that we make real impact.
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*in combination with conventional therapy.