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Oscillatory behavior of $P$ wave duration and $PR$ interval in experimental congestive heart failure: a preliminary study

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**Keywords:** chronic heart failure, $P$ wave, autonomic nervous system, arrhythmias, vagal nerve activity (VNA), stellate ganglion nerve activity (SGNA), heart rate variability (HRV)

**Abstract**

**Objective:** The relationship between the autonomic nervous system (ANS) modulation of the sinus node and heart rate variability has been extensively investigated. The current study sought to evaluate, in an animal experimental model of pacing-induced tachycardia congestive heart failure (CHF), a possible ANS influence on the $P$ wave duration and PR interval oscillations. **Approach:** Short-term (5 min) time and frequency domain analysis has been obtained in six dogs for the following electrocardiographic intervals: $P$ wave duration ($P$), from the onset to peak of $P$ wave ($P_p$), from the onset of $P$ wave to the $q$ onset ($PR$) and from the end of $P$ wave to the onset of $q$ wave ($P_R$). Direct vagal nerve activity (VNA), stellate ganglion nerve activity (SGNA) and electrocardiogram (ECG) intervals have been evaluated contextually by implantation of three bipolar recording leads. **Main results:** At the baseline, multiple regression analysis pointed out that VNA was strongly positively associated with the standard deviation of $PP$ and $PR$ intervals ($r^2: 0.997, p < 0.05$). The same variable was also positively associated with high-frequency (HF) of $P$ expressed in normalized units, of $P_p$ and of $PR$ ($b: 0.001$) ($r^2: 0.993; p < 0.05$). During CHF, most of the time and frequency domain variability significantly decreased from 20% to 50% in comparison to the baseline values ($p < 0.05$) and SGNA correlated inversely with the low frequency (LF) obtained from $PR$ ($p < 0.05$) and $PR$ ($p < 0.05$) ($r^2: 0.899, p < 0.05$). LF components, expressed in absolute and normalized power, obtained from all studied intervals, were reduced significantly during CHF. Any difference between the $RR$ and $PP$ spectral components was observed. **Significance:** The data showed a significant relationship between ANS and atrial ECG variables, independent of the cycle duration. In particular, the oscillations were vagal mediated at the baseline, while sympathetic mediated during CHF. Whereas $P$ wave variability might have a clinical utility in CHF management, it needs to be addressed in specific studies.

**Introduction**

It is well known that acute or chronic increase of left ventricular end-diastolic pressure, induced by several pathological conditions, could fatally induce a change in both atrial structure and function. Accordingly, in chronic heart failure (CHF) (Joung et al 2010a, 2010b) the electromechanical atrial remodeling, the sinus node dysfunction and supraventricular arrhythmias have been described. During healthy condition, the autonomic nervous system (ANS) modulates the sinus node activity, leading to the well-known oscillatory behavior of heart rate, due to the opposite and alternating vagal and sympathetic activity. Conversely, several cardiovascular...
diseases are often associated with ANS imbalance with a sympathetic over-activity, which in turn is able to provoke a significant reduction of the heart rate variability (HRV) (Mortara et al 1994, Guzzetti et al 1995, Piccirillo et al 2006, 2009a). Thus, the oscillatory activity of the sinus node constantly and indirectly may provide useful insight into atrial and ventricular functions (Piccirillo et al 2016a, 2016b). Oscillatory activity originates in the sinus node and it spreads to the atria, the atrioventricular node and then to the ventricles. Although the RR interval variability is thought to be an exact surrogate of the PP interval variability, these two electrocardiographic intervals pertain to different cardiac chambers. Indeed, both atrial and ventricular damage could affect the supraventricular conduction system and hence the PP oscillations precede temporally those of the RR intervals (Horner et al 1996). The abovementioned extreme sensitivity of the supraventricular conduction systems to possible structural and functional heart changes raises interest in investigating possible earlier markers of ANS imbalance.

The previous studies on the HRV during CHF, focusing on the RR intervals, reported an RR variability reduction, expressed as time and frequency domain variables in short- and long-period electrocardiogram (ECG) recordings. In particular, in CHF the reduction of an RR spectral component, called low-frequency (LF) power, was related to the increase of left ventricular end diastolic pressure (Horner et al 1996), sinus dysfunction (Piccirillo et al 2009b), clinical progression of disease (Mortara et al 1994, Guzzetti et al 1995, Van de Borne et al 1997, Piccirillo et al 2004, 2006, Sanders et al 2004) and, moreover, the risk of sudden cardiac death (La Rovere et al 2003) and mortality (Guzzetti et al 2005) in both human and experimental models.

Obviously, the RR and PP power spectral analysis showed similar results and were then extensively studied, but we have few data on the intrinsic oscillations of the P wave and PR segment in CHF and in relation to autonomic control. Previous observations showed a reduction of PQ–PP and P–PP spectral coherence in CHF patients in comparison with normal subjects; these data could indicate atrial distress during an increase of left ventricular end-diastolic pressure and atrial enlargement.

The aim of this study was to evaluate the influence of ANS on the P wave and PR interval oscillations in CHF in order to understand if these variables could be used as a marker of autonomic influence, compared to the direct recording of stellate ganglia and vagal nerve activities (VNAs), on sinus and atrioventricular nodes.

Materials and methods

Surgical preparation and electrical recording

The data analyzed came from a previous study conducted in six female dogs (Ogawa et al 2007, Choi et al 2010, Han et al 2012). The surgical procedures and temporal relationship between cardiac arrhythmia and ANS activity are reported in detail elsewhere (Ogawa et al 2007, Choi et al 2010, Han et al 2012). Briefly, a pacing lead was implanted in the right ventricular apex and connected to an Itrel neurostimulator (Medtronic, Minneapolis, MN, USA) in a subcutaneous pocket. We then implanted a Data Sciences International (DSI) D70-EEE transmitter with three bipolar recording channels for simultaneous recording of the left stellate ganglion nerve activity (SGNA), VNA from the left thoracic vagal nerve located above the aortic arch, and subcutaneous ECG. After implantation, the Itrel stimulator was initially turned off for two weeks to allow the dogs to recover from surgery and to allow us to obtain the baseline recordings. The stimulator was then programmed to pace at 150 bpm for 3 d, at 200 bpm for 3 d, and then at 250 bpm for 3 weeks to induce CHF. The pacemaker was then turned off to allow an additional two weeks of ambulatory monitoring and recording during CHF. All CHF data were recorded within the first week. All animals underwent the same protocol with regard to intensity and duration of pacing. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Cedars-Sinai Medical Center, Los Angeles, California.

Direct measurement of autonomic nervous activity

Data were recorded in real time at a sampling rate of 1000 samples s⁻¹ per channel, then analyzed off-line. The software used has been described elsewhere (Ogawa et al 2007, Choi et al 2010, Han et al 2012). In brief, to analyze long-term trends in the large segmented data files effectively, a custom-designed program was developed using Labview™ (National Instruments, Austin, Texas, USA) software to automatically import, filter and analyze the DSI transmitter data for ANS activities and heart rates. The software determined the activation cycle length (RR intervals) automatically derived from the ECG (based on a Hilbert transform algorithm (Benitez et al 2001). Integrate data from the SGNA and VNA were high-pass (200 Hz) filtered and rectified over a fixed time segment to represent the total nerve activity. Because RR intervals shorter than 200 ms were usually due either to ectopic beats, artifacts or rhythm disturbances, they were removed and excluded from the analysis.

To assess ANS activity, we randomly selected six short-term recordings (5 min ECG) for each dog at the baseline and after pacing-induced CHF. Moreover, nerve activity data were also calculated over 24 h, namely in a total of 288 of 5 min epochs; finally, we calculated the means of these data and reported them as 24 h SGNA and VNA.
The coherence function provides a measure between zero and unity in the degree of linear interaction between the different studied interval oscillations as a function of their frequency. Mean coherences were measured by averaging $\gamma (f)$ over the frequency bands: from 0–0.50 Hz.

To detect the ECG intervals, we used a classic adaptive first derivative/threshold algorithm, explained elsewhere (Arzeno et al 2008). Also, to acquire, store and analyze the ECG data, we used our designed and produced software using Labview™ (National Instruments, Austin, Texas, USA). A cardiologist (GP) checked the different ECG wave and intervals, automatically marked by the software, and, when needed, manually corrected the mistakes. The premature beats and excessive noise were causes of possible mistakes in the interval measurement, so we have not considered in the analysis recordings with more than three premature beats.

HRV power spectral analysis

We calculated the following intervals from 5 min epochs of ECG recordings: RR, PP, P wave (from onset to end of P wave), PR (from the onset of P wave to onset of q wave), and $P_q$ (between the onset and the peak of P wave) and $P_{pR}$ (from the end of P wave to the onset of q wave) intervals (figure 1).

![Figure 1. $P_p$ (1), $P,R$ (2), $PR$ (4), $PP$ (5), $RR$ (6) intervals and $P$ (3) wave duration.](image)

We measured the mean, variance and standard deviation (SD) values for each interval. In particular, the value obtained for each variable was the mean of six-sample measurements before and after pacing-induced CHF, in the six dogs studied.

We used an autoregressive algorithm to compute the power spectral density from the different ECG variables (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). From a series of 5 min of consecutive beats, the autocorrelation function was applied to the complete RR, $P_p$, PR, $P$, $P_{pR}$, and PP series, and the Yule–Walker matrix (autocorrelation value matrix) was calculated with the Levinson–Durbin method; the matrix order was determined with an Anderson test and Akaike Information Criteria. The spectral decomposition method was then applied for the estimation of power and central frequency of each spectral component. Spectral power of each variable is expressed in milliseconds squared.

The frequencies of the RR, $P_p$, PR, $P$, $P_{pR}$, and PP oscillations divided by the mean RR, $P_p$, PR, $P$, $P_{pR}$, and PP values, respectively expressed in milliseconds, were calculated in cycles per beat and converted to herz equivalents. We then determined the following power spectral variables in absolute power expressed in milliseconds squared of the RR, $P_p$, PR, $P$, $P_{pR}$, and PP intervals: total spectral power (TP) or root square of variance are expressed as SD; the high-frequency (HF) component (from 0.15–0.40 Hz); LF component (from 0.04–0.15 Hz) (Piccirillo et al 2004, 2009a, Magri et al 2012).

Thereafter, the coherence function for the RR and the various repolarization intervals was estimated (Piccirillo et al 2009a, 2016a, 2016b, Baumert et al 2016). Coherence expresses the power fraction oscillating at a given frequency in either time series and is explained as a linear transformation of the other, thus indicating a linear association between the two signals. The coherence function $\gamma (f)$ was then computed according to the formula:

$$\text{Coherence} (f) = \frac{|P_{xy}(f)|^2}{P_{xx}(f)P_{yy}(f)}$$

where $f$ is frequency, $P_{xx} (f)$ is the spectrum of an obtained variable ($PR$, $P_p$, $P$ or $RR$), $P_{yy} (f)$ are the other studied spectral variables ($PP$, $PR$, or $P_{pR}$), and $P_{xy} (f)$ is the cross-spectrum ($PR$–$PP$, $P_q$–$PP$, $P$–$PR$, $P_{pR}$–$PR$, or $RR$–$PP$).

The coherence function provides a measure between zero and unity in the degree of linear interaction between the different studied interval oscillations as a function of their frequency. Mean coherences were measured by averaging $\gamma (f)$ over the frequency bands: from 0–0.50 Hz.

To detect the ECG intervals, we used a classic adaptive first derivative/threshold algorithm, explained elsewhere (Arzeno et al 2008). Also, to acquire, store and analyze the ECG data, we used our designed and produced software using Labview™ (National Instruments, Austin, Texas, USA). A cardiologist (GP) checked the different ECG wave and intervals, automatically marked by the software, and, when needed, manually corrected the mistakes. The premature beats and excessive noise were causes of possible mistakes in the interval measurement, so we have not considered in the analysis recordings with more than three premature beats.

At the baseline and after the pacing-induced CHF, the left ventricular ejection fraction, by echocardiographic study, and plasma levels of norepinephrine N-terminal and pro-B type natriuretic peptide (NT-proBNP) were evaluated.

Statistical analysis

Unless otherwise indicated, all data are expressed as mean ± SD. Data with skewed distribution are given as median and interquartile range (75th percentile—25th percentile).
After pacing-induced CHF, in all six dogs, we obtained a significant reduction of ejection fraction \( p < 0.0001 \) and a significant increase of norepinephrine \( (p < 0.01) \) and NT-proBNP levels in peripheral blood flow \( (p < 0.05) \) (Ogawa et al 2007). The voltage of the \( P \) wave was not significantly different between the baseline and CHF.

During the CHF period, the SDs of all variables \( (RR, PP, P_p, P_R) \) showed a significant reduction in comparison with the baseline period \( (p < 0.05) \) (table 1).

Similarly, the TP of each variable was lower during CHF. Finally, during CHF, \( PR–PP, P_p–PP, \) and \( P–P_R \) spectral coherences were lower than at the baseline condition \( (p < 0.05 \) for all) (table 1). Conversely, both the 24 h SGNA \( (p < 0.0001) \) and 24 h VNA were significantly higher \( (p < 0.0001) \) during CHF (table 1).

\( LF \), expressed in absolute power, was significantly lower during CHF in all studied variables \( (P_p, P_R, PR, P, P_pR, P_R, P) \) (figure 2).

\( LF \), expressed in normalized units (NU$s$), confirmed the same behavior only for \( P_p \) and \( PR \) (figure 3).

Conversely, only HF absolute power of \( P \) was significantly lower during CHF in comparison with the baseline (baseline median: 42 ms$^2$, interquartile range: 46 ms$^2$ versus CHF median: 21 ms$^2$, interquartile range: 10 ms$^2$). We did not find any difference in the matrix order between the baseline and CHF period (baseline 17 ± 8 versus CHF: 20 ± 6, \( p \) ns). We did not observe any difference between the \( RR \) and \( PP \) spectral components.

### Table 1. ECG variables at the baseline and during CHF.

| Variables          | Baseline N = 6 | CHF N = 6 | \( P \) values |
|--------------------|----------------|-----------|----------------|
| \( RR \) mean, ms  | 768 ± 66       | 651 ± 97  | NS             |
| \( RR \) SD,       | 300 ± 44       | 160 ± 48  | 0.009          |
| \( PP \) mean, ms  | 767 ± 67       | 651 ± 96  | NS             |
| \( PP \) SD,       | 305 ± 40       | 161 ± 47  | 0.006          |
| \( P_p \) mean, ms | 48 ± 17        | 49 ± 19   | NS             |
| \( P_p \) SD,      | 9 ± 3          | 7 ± 2     | 0.032          |
| \( PR \) mean, ms  | 149 ± 30       | 143 ± 23  | NS             |
| \( PR \) SD,       | 7 ± 2          | 5 ± 2     | 0.020          |
| \( P \) mean, ms   | 95 ± 26        | 91 ± 25   | NS             |
| \( P \) SD,        | 10 ± 3         | 8 ± 2     | 0.047          |
| \( P_pR \) mean, ms| 54 ± 9         | 53 ± 8    | NS             |
| \( P_pR \) SD,     | 9 ± 2          | 4 ± 2     | 0.000          |
| \( PR–PP \), coherence | 0.275 ± 0.111 | 0.161 ± 0.028 | 0.048        |
| \( P_p–PP \), coherence | 0.317 ± 0.075 | 0.223 ± 0.069 | 0.030        |
| \( P–PR \), coherence | 0.564 ± 0.126 | 0.654 ± 0.111 | NS            |
| \( P–P_pR \), coherence | 0.666 ± 0.065 | 0.488 ± 0.167 | 0.014          |
| \( RR–PP \), coherence | 0.970 ± 0.038 | 0.961 ± 0.030 | NS            |
| SGNA (µV/sample)  | 0.29 ± 0.20    | 0.43 ± 0.24 | 0.039          |
| VNA (µV/sample)   | 0.11 ± 0.02    | 0.14 ± 0.06 | NS             |
| SGNA 24 h (µV/sample) | 0.47 ± 0.09   | 0.62 ± 0.31 | 0.0001         |
| VNA 24 h (µV/sample) | 0.009 ± 0.003 | 0.026 ± 0.02 | 0.0001         |

Data are expressed as mean ± SD. SGNA: integrated left stellate ganglion nerve activity; VNA: integrated vagal nerve activity. NS: not significant.

In the present study, we analyzed six ECG registrations for the dogs at the baseline and after pacing-induced CHF. Therefore, we used the mean of six values for each variable for the statistical analysis at the baseline and after pacing-induced CHF. In conclusion, each variable was measured 36 (six dogs for six recordings) times before and after pacing-induced CHF.

The paired \( t \)-test was used to compare data for the normally distributed variables (including mean and SD of ECG variables, coherence functions, etc), whereas the Wilcoxon test was used to compare non-normally distributed variables (including variance and spectral components in absolute power of \( RR, P_p, PR, P, P_pR, \) and \( PP \) intervals and plasma levels of norepinephrine and NT-proBNP) at the baseline and after CHF. A stepwise multiple regression analysis was constructed to study possible associations between variables. In particular, we consider SGNA or VNA as a dependent variable and spectral and non-spectral \( (RR, P_p, PR, P, P_pR, \) and \( PP \) studied data as independent variables at the baseline and after pacing-induced CHF.

### Results

In conclusion, each variable was measured 36 (six dogs for six recordings) times before and after pacing-induced CHF.
With regard to short-term time and frequency domain data, the stepwise multiple regression analysis showed a significant inverse relation at the baseline between SGNA and $P_e$ RSDs ($r: 0.937; b: -0.180, p < 0.05$) and the HF of $P$ ($r: 0.912; b: -0.016, p < 0.05$), expressed in NUs (figure 4).

In contrast, the VNA was positively associated with SD of $P_P$ intervals ($r: 0.9998, p < 0.05$) (figure 5(A)). The same nerve variable was also positively associated with the HF of $P$ expressed in NUs ($b:0.002$), of $P_P$ ($b:0.000$), and of $P_e$ R ($b:0.001$) ($r:0.998; p < 0.05$) (figure 5(B)).

Conversely, during CHF, the stepwise multiple regression analysis showed just a significant inverse relation between SGNA and the LF of $P_e$ R, expressed in NUs, ($r:0.948; b: -0.027, p < 0.01$) (figure 6).

**Discussion**

The major and novel finding of the study was that pacing-induced tachycardia CHF produced a reduction of the temporal variability of the P wave duration and of $P_e$ $P_P$, $P_e$ R, and PR intervals. In particular, all the short-term atrial ECG time-variables showed a reduction, expressed in SD, in CHF. Contextually, during CHF, a global reduction of LF power has been observed.

In fact, changes in the ANS modulation of the sinus node autonomic, together with a neuro-humoral and inflammatory activation, are common CHF features possibly related to an increased risk of atrial arrhythmias. A simultaneous increase in sympathetic and VNA has been described at the atrial level (Choi et al 2010, Han et al 2012), with the vagal activity increase being most likely a compensatory attempt to reduce the final sympathetic
hyperactivity. The abovementioned ANS imbalance leads to an increased temporal dispersion of refractory periods of the atrial myocytes, which in turn represents a trigger for atrial arrhythmias (i.e. atrial fibrillation). The observed reduction of variability of the atrial related ECG parameters during CHF, similar to those of the RR variability (Piccirillo et al 2009b), could be the direct consequence of this peculiar and proarrhythmic autonomic control (Piccirillo et al 2016a, 2016b). Interestingly, the reduced variability of these atrial variables was independent of the heart rate in this new experimental model (ECG recordings with similar heart rate analyzed) (Zaza and Lombardi 2001). In fact, in the pacing-induced CHF condition, we observed an equal level of VNA compared with the baseline even at the same heart rate. On the other hand, we found a higher VNA level on 24 h nerve recordings during CHF. Thus, although the higher levels of VNA could seem a paradox, it seems to be a compensatory mechanism to oppose the increase of SGNA (Choi et al 2010, Ogawa et al 2010, Han et al 2012).

Further support to this VNA behavior, comes from the CHF-related upregulation of the muscarinic receptors (M2) reported in both human and animal models (DeMazumder et al 2015). Furthermore, this increase of vagal activity could be interpreted as an increase of vagal afferent activity related to the increase of end diastolic pressure and atrial distention in CHF.

Moreover, we observed that the VNA influenced prevalently the SD (time domain variables) of the $P_R$ and $PP$ intervals and the HF (frequency domain variables) of the $P$ wave duration at the baseline. Conversely, the
SGNA had no influence on any atrial time domain variables. This finding is not unexpected, because the HRV expressed as SD in short-term ECG recordings is a marker of vagal activity (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996) and, hence, the positive relation between VNA and the \( P_R \) interval SD confirms that this parameter could be considered a noninvasive marker of vagal atrial activity under healthy condition. In fact, undisputed specific papers in this field, albeit dated, reported that the vagal pharmacological suppression induces a dramatic reduction of the short period of SD of RR and for this reason, all spectral components (Pomeranz et al 1985, Koh et al 1994, Després et al 2002, Laude et al 2008). Finally, this reduction was found to be abolished in the absence of VNA (Epstein et al 1990). In addition, our data suggest that these atrial variables are probably more reliable than RR variability for sinus vagal activity (i.e. stepwise multivariable analysis did not show a significant relation between RR SD and the VNA).

Indeed, atria, sinus node and atrioventricular node are extensively innervated by parasympathetic nerves. Thus, we could consider that particularly the \( P_R \) interval represents the best noninvasive surrogate of the electrical conduction between the atrioventricular node and the His bundle. On the other hand, the atrial spectral markers,
that is the HF of $P_R$ and $P$, could express the effect of vagal influence on the right and left atria. In fact, the first part of the $P$ wave, since the $P$ peak is probably linked to the electrical depolarization of the right atrium (Puech et al. 1974), whereas the total $P$ wave duration is mainly influenced by the left atria. (Chirife et al. 1975). In conclusion, these spectral variables were markers of vagal effect on both atria in normal condition (figure 5).

In power spectral and time domain atrial variables related to the SGNA, the influence seems to be partially coincident in baseline and CHF conditions. In fact, we found an inverse relation between SGNA and $P_R$ vagal-derived markers in both these experimental conditions. In particular, we found that SD and HF of $P_R$ are characterized by an inverse relationship, expressed in NUs. This datum could indicate that the sympathetic activity, recorded in the left stellate ganglion, induced a reduction of vagal activity in the atrioventricular node and in His bundle. Naturally, a large and historical pathophysiological literature reported that left side sympatho-vagal innervations, as in our study, had a prevalent influence on atrioventricular node function (Cohn et al. 1993, Cinca et al. 1985, Inoue et al. 1987, Waninger et al. 1999).

The inverse relationship between the left SGNA and VNA seemed to have only one possible and easy explanation: sympathetic activation was able to reduce the vagal activity in atrioventricular node and in His bundle in normal and CHF condition, inducing the well-known phenomenon called sympathetic-parasympathetic interaction in the A–V conduction system (Salata et al. 1986, Levy et al. 1988, Brack et al. 2004, White et al. 2014).

Although the physiological meaning of LF still remains controversial (Parati et al. 1995, Sleight et al. 1995, Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996, Moak et al. 2007, Piccirillo et al. 2009a), the Mayer waves, that cause these oscillations (about 0.10 Hz), are reduced in the CHF for the $P_R$ and PR segments in the same way as RR variability (van de Borne et al. 1997, Piccirillo et al. 2006). Moreover, both in human and in experimental CHF models, the LF reduction is related to severity of the disease and it is a marker of CHF-related mortality (Mortara et al. 1994, van de Borne et al. 1997, La Rovere et al. 2003, Guzzetti et al. 2005, Piccirillo et al. 2009b). Thus, the LF reduction of atrial variables could be considered a noninvasive and inexpensive marker of the degree of dysfunction of the atrial and ventricular myocardium.

In conclusion, the atrial ECG-derived intervals showed oscillations similar to those of the RR interval and, in normal conditions, they seem to be controlled primarily by VNA. In contrast, during experimental CHF, the atrial ECG-derived intervals collapse dramatically, with these reductions related to increased sympathetic nerve activity. Thus, different atrial structures seemed to have different oscillatory behavior according to the autonomic control and the presence of CHF. The $P$ wave duration and PR interval oscillation evaluation might be a stimulating idea for future research on paroxysmal supraventricular arrhythmias. Moreover, given that the ECG yields a simple and transmissible signal, this easy approach could be developed as a remote medical technology, thereby saving time and economic resources.

Study limitation
The most important study limitation was the small number of studied animals. However, it should be emphasized that we checked every point automatically found by the software for a total of about 150 000 points (five points for each beat). Analyzable recordings should be of good quality, avoiding confounding noise (resulting from environmental conditions, i.e. dog movements). Thus, we analyzed the study variables in 36 5 min ECG recordings at the baseline and after pacing-induced CHF (six baselines and six CHFs for each of the six cases). The abovementioned vast amount of work, which took nearly two years, led us to obtain measurements with a high level of accuracy.

Another possible limitation was that the HRV, SGNA and VNA were markers of ANS modulation as mean in 5 min rather than a marker of a moment-to-moment or instantaneous nerve activity. Because the HRV needed a stationary period (see figure 3), we also possibly choose a stationary period of nerve activity, and therefore the HRV, SGNA and VNA were numerically expressed as mean of 5 min.

The last technical limitation of direct nerve recording was undoubtedly that the adopted approach did not permit us to discriminate the afferent from efferent nerve activity. Therefore, we need to consider the possibility to have a pure efferent cardiovascular signal.

Conflict of interest
The authors declare no financial or industrial interests regarding this study.

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