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Multi-lag tone–entropy in neonatal stress

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Heart rate variability (HRV) has been analysed using linear and nonlinear methods. In the framework of a controlled neonatal stress model, we applied tone–entropy (T–E) analysis at multiple lags to understand the influence of external stressors on healthy term neonates. Forty term neonates were included in the study. HRV was analysed using multi-lag T–E at two resting and two stress phases (heel stimulation and a heel stick blood drawing phase). Higher mean entropy values and lower mean tone values when stressed showed a reduction in randomness with increased sympathetic and reduced parasympathetic activity. A ROC analysis was used to estimate the diagnostic performances of tone and entropy and combining both features. Comparing the resting and simulation phase separately, the performance of tone outperformed entropy, but combining the two in a quadratic linear regression model, neonates in resting as compared to stress phases could be distinguished with high accuracy. This raises the possibility that when applied across short time segments, multi-lag T–E becomes an additional tool for more objective assessment of neonatal stress.

1. Introduction

Neonatal stress is a special field of research and clinical practice that can be considered from a wide spectrum of perspectives [1–3]. Endocrinologically, during a stress phase, there is a large excretion of hormones generated to cope with a potentially life-threating event, further activating a part of the autonomic nervous system (ANS), and producing significant changes in every body system—basically changing the body's homeostasis [4–6]. These described changes are a result of the activation of the sympathetic branch of the ANS, which can result in an increase in the heart and respiratory rate, blood pressure, causing an increase of stress hormone levels (i.e. adrenaline, noradrenaline, cortisol, etc.). By contrast, the parasympathetic branch predominates, when there is no need for an acute stress response. The activation of the parasympathetic branch of the ANS decreases the heart and respiratory rate, as well as blood pressure, increases salivation, urination, digestion, defecation, all of which should be delayed in acute stress situations [7,8]. Owing to the constant interaction with various stimuli, the perturbations in the predominance of one of the two branches of the ANS constantly occurs, trying to maintain homeostasis. However, imbalances, such as prolonged activation of the sympathetic or inhibition of the parasympathetic branch, are considered risk factors in various diseases, which can ultimately lead to death (i.e. ventricular tachyarrhythmias and sudden cardiac death) [9,10].

Clinically, neonates and especially those born prematurely can sometimes present with vague clinical signs and symptoms of disease [11–14]. As children physiologically differ from adults, stressors that do not affect adult humans

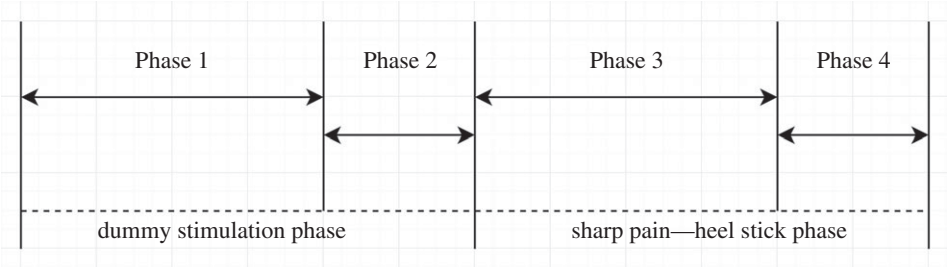

Figure 1. The sequence of phases used in the study protocol. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 3, second baseline phase; Phase 4, heel stick blood drawing phase.

might have a significant impact on the very young, altering their long-term development [15,16]. In addition to environmental stressors naturally characteristic of the neonatal intensive care unit (NICU), (i.e. noise, temperature, etc.) if the neonate is further challenged with disease or early gestational age at birth, they experience more stress from various invasive medical procedures they undergo [17].

Heart rate variability (HRV) is the variability of the duration between successive cardiac cycles that originate in the sinus node [18]. HRV has broad application in basic ANS science and clinical use via the application of different mathematical and statistical methods: time and spectral analysis, measures of entropy, Hurst exponents, indices derived from chaos theory such as the correlation dimension, Poincaré plots, recurrence plots or Lyapunov exponents [19–22].

Among the various HRV parameters, tone–entropy (T–E) showed promising results in identifying patients with ANS dysfunction [23]. T–E uses successive RR intervals with the implicit assumption that the current heartbeat is influenced by the immediately preceding beat. However, it has been reported that each heartbeat influences not only the beat immediately following it, but also up to 6–10 beats downstream, possibly as a consequence of respiratory sinus arrhythmia and baroreflex influence [24]. In addition, several researchers have shown that the delayed signal can be used for measuring the influence on the succeeding beats [25] and the delay is associated with the disease processes [24]. Thus, successive RR interval duplets will likely underestimate the role of the autocovariance function of RR intervals, i.e. the ability of heart beats to influence a train of succeeding beats. The strong correlation between successive beats (in case of lag 1 T–E) also masks the nonlinearity from the measurement [23,26]. Thus, use of multi-lag analysis can enable the autocovariance function to capture a nonlinear aspect of HRV. In a previous study [19], it has been reported that multi-lag T–E analysis can overcome the limitations of the single-lag T–E analysis in HRV studies. They showed that multi-lag T–E analysis better stratified the risk of autonomic neuropathy in Type 2 diabetes mellitus than single-lag T–E [19]. In addition, a rationale for the use of T–E relies on the evidence that T–E is not influenced by the period of data collection or the baseline heart rate (HR) [27].

2. Subjects and methods

2.1. The study protocol

Forty healthy term neonates (21 females and 19 males, birth weight 3542.05 ± 339.09 g), all of who were born through vaginal delivery, with an APGAR score >9/9 at 1 and 5 min, without perinatal risk factors, breastfed or additionally formula fed if not enough breastmilk was supplied, and not previously experiencing any external stress stimuli, were included in this study. The neonates were chosen by simple random sampling over a time period from 30 September 2016 to 10 February 2017, in the paediatrics clinic's maternity ward of the Osijek University Clinic. Among the participants, only two female neonates were twins, while the rest of the participants were singletons. The average gravidity and parity of the participant's mothers were 1.66 ± 0.92. All mothers were euthyroid, one participant's mother was diagnosed with gestational diabetes, but did not require treatment with insulin therapy, and none of the mothers had prior recorded miscarriages. The experimental paradigm was performed at the recommended chronological age for routine metabolic screening, 72 h of age, just prior discharge [28,29]. None of the neonates included in this study underwent any other procedural pain besides the national metabolic screening heel stick blood draw. As we have described previously [30], the protocol is divided into three parts: (a) dummy stimulation, (b) the sharp pain—heel stick, (c) the treatment; only the first two parts were included in the study (figure 1).

Part (a) consisted of two phases: the first, the baseline phase (no stimulation), lasting 10 min (further named Phase 1), after which intermittent pressing of the neonate's heel was done, mimicking a heel stick blood drawing procedure without actual blood sampling (Phase 2). Phase 2 lasted 90 s, which is the average time for the nurse to perform the blood drawing procedure. The end of Phase 2 is the starting point of Part (b). Part (b) also consisted of two phases: Phase 3, the second baseline (no stimulation), again lasting 10 min, followed by the actual heel stick blood sampling procedure (Phase 4).

A lightweight, high-resolution device, with a high sampling rate (1024 Hz) was used (Firstbeat Bodyguard 2, Firstbeat Technologies Ltd, Jyvaskyla, Finland) during the entire procedure. After visual inspection and artefact removal, the data were analysed. To reduce movement artefacts and ensure quality of the recordings, the neonates were positioned supine after breast- or formula feeding in a quiet room.

2.2. Multi-lag T–E analysis of HRV signal

The RR interval is defined as the time difference between two consecutive R peaks of the electrocardiogram signal and the RR intervals in time series (RR_i) are defined as

$$RR \equiv (RR_1, RR_2, \ldots, RR_N),$$

Table 1. Mean ± s.d. of tone values across the different phases of the protocol with varying lags. *P*-values were calculated using one-way ANOVA, and represent significance of differences among tone values obtained at different phases. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 3, second baseline phase; Phase 4, heel stick blood drawing phase.

lag	Phase 1	Phase 2	Phase 3	Phase 4	*P**
1	−0.09 ± 0.10	0.04 ± 0.09	−0.09 ± 0.11	−0.02 ± 0.13	<0.001
2	−0.12 ± 0.10	0.11 ± 0.18	−0.12 ± 0.11	0.00 ± 0.19	<0.001
3	−0.17 ± 0.12	0.19 ± 0.27	−0.17 ± 0.15	0.03 ± 0.26	<0.001
4	−0.20 ± 0.13	0.26 ± 0.37	−0.21 ± 0.17	0.05 ± 0.34	<0.001
5	−0.24 ± 0.15	0.34 ± 0.47	−0.24 ± 0.20	0.08 ± 0.42	<0.001
6	−0.27 ± 0.16	0.42 ± 0.56	−0.27 ± 0.22	0.11 ± 0.51	<0.001
7	−0.31 ± 0.17	0.49 ± 0.66	−0.30 ± 0.24	0.14 ± 0.60	<0.001
8	−0.33 ± 0.18	0.57 ± 0.76	−0.33 ± 0.26	0.17 ± 0.70	<0.001
9	−0.35 ± 0.19	0.64 ± 0.86	−0.35 ± 0.28	0.21 ± 0.81	<0.001
10	−0.38 ± 0.20	0.72 ± 0.96	−0.36 ± 0.29	0.24 ± 0.91	<0.001

where N is the number of RR_i. HR acceleration and inhibition can be determined from the difference of consecutive RR_i. If RR_{i+1} becomes shorter than RR_i, then it is an acceleration of HR. Therefore, acceleration of the heart is expressed as a plus difference and inhibition as a minus difference of RR_i. However, to reduce the impact of HR variation over a wide range of time and different subjects, normalized variation in RR_i is preferred to monitor the variability. This normalized variation is measured by percentile change (percentage index (PI)) and defined as:

$$PI_i^m = \frac{RR_i - RR_{i+m}}{RR_i} \times 100, \tag{2.1}$$

where m is an integer and represents the lag used for measuring the PI. The detailed methodology of multi-lag T–E analysis has been described in a previous report [23]. The *Tone* at lag m ($Tone^m$) is defined as a first-order moment (arithmetic average) of this $\mathbf{PI^m}$ time series as

$$Tone^m = \frac{1}{N-m} \sum_{i=1}^{N-m} PI_i^m. \tag{2.2}$$

The *Entropy* at lag m is defined from the probability distribution of $\mathbf{PI^m}$ by using Shannon's formula [31]:

$$\text{Entropy}^m = -\sum_{i=1}^{n} p(i) \log_2 p(i), \tag{2.3}$$

where $p(i)$ is a probability that PI_n^m has a value in the range $i \leq PI_n^m < i+1$, i is an integer. The Entropy^m evaluates total acceleration–inhibition activities, or total heart period variations, in a familiar unit of bit.

2.3. Statistical analysis

The data were analysed with the Matlab R2007a software. The Kolmogorov–Smirnov test was used to test the normality of distributions. The data are descriptively presented with means and standard deviations. A repeated measures ANOVA, followed by a pairwise post hoc test, was applied to assess the mean differences of tone and entropy across the different phases. An ROC curve analysis was used to test the diagnostic properties of the T–E comparing the stress phases (Phase 2 and 4) to the first baseline (Phase 1). The results of the ROC curve analysis are presented as the total area under the ROC curve (AUC), which represents the performance of the model. AUC values enable comparison of the performance of different models, and it is generally accepted that models with higher AUC values have a better overall performance of correctly classifying diseased (in our case stress phase) from non-diseased (resting phase) subjects. *P*-values less than 0.05 were considered statistically significant.

3. Results

There were statistically significant differences ($p < 0.001$) when comparing both the tone and entropy values across the four phases during the first 10 lags (tables 1 and 2). Over the varying lags, at the resting phases (Phase 1 and 3), a steady linear decrease of tone values was observed ranging from −0.09 ± 0.10 in Phase 1, and −0.09 ± 0.11, at lag 1, up to −0.38 ± 0.20 in Phase 1, and −0.36 ± 0.29 (Phase 3) (table 1). On the contrary, at both stress phases (Phase 2 and Phase 4), a steady linear increase in the tone values is observed. The tone values at Phase 2 were always positive, being the lowest at lag 1 (0.04 ± 0.09), and highest at lag 10 (0.72 ± 0.96), while a single negative tone value was observed at lag 1 of Phase 4 (−0,02 ± 0.13). At every lag the tone values of Phase 4, were lower than in Phase 2, being highest at Phase 4 (0.24 ± 0.91).

Contrary to the tone values which decrease in the stress phases (table 1), a steady increase of entropy is observed at all phases (table 2). At each lag, the entropy values were observed to be lower at stress (Phase 2 and 4) than at rest (Phase 1 and 3). The lowest entropy at all phases was observed at lag 1 (Phase 1: 3.63 ± 0.63, Phase 2: 3.11 ± 0.59, Phase 3: 3.58 ± 0.74, Phase 4: 4.28 ± 0.60), while the highest values were observed at lag 10 (Phase 1: 4.87 ± 0.46, Phase 2: 4.26 ± 0.59, Phase 3: 4.26 ± 0.59, Phase 4: 4.28 ± 0.60).

The post hoc *p*-values comparing both tone and entropy across the phases are presented in table 3. When comparing both tone and entropy values in the resting phases (Phase 1 and Phase 3), no significant differences were observed. Tone significantly differed between Phase 1 and 2 (*p*-values: from lag 1 to lag 10 − <0.001), and Phase 1 and 4 at all lags (*p*-values: lag 1 – 0.015, lag 2 – 0.001, from lag 3 to lag 10 − <0.001), as well as between Phase 3 and Phase

Table 2. Mean ± s.d. of entropy values across the different phases of the protocol with varying lags. *P*-values were calculated using one-way ANOVA, and represent significance of differences among entropy values obtained at different phases. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 3, second baseline phase; Phase 4, heel stick blood drawing phase.

lag	feature	Phase 1	Phase 2	Phase 3	Phase 4	*P**
1	entropy	3.63 ± 0.63	3.11 ± 0.59	3.58 ± 0.74	3.22 ± 0.59	<0.001
2	entropy	3.96 ± 0.56	3.46 ± 0.59	3.93 ± 0.66	3.51 ± 0.58	<0.001
3	entropy	4.23 ± 0.53	3.65 ± 0.59	4.18 ± 0.65	3.69 ± 0.59	<0.001
4	entropy	4.39 ± 0.51	3.80 ± 0.59	4.35 ± 0.63	3.85 ± 0.60	<0.001
5	entropy	4.54 ± 0.49	3.93 ± 0.57	4.47 ± 0.63	3.96 ± 0.61	<0.001
6	entropy	4.63 ± 0.48	4.02 ± 0.59	4.57 ± 0.62	4.05 ± 0.60	<0.001
7	entropy	4.72 ± 0.47	4.08 ± 0.59	4.64 ± 0.61	4.12 ± 0.61	<0.001
8	entropy	4.77 ± 0.47	4.15 ± 0.59	4.70 ± 0.60	4.18 ± 0.60	<0.001
9	entropy	4.82 ± 0.46	4.21 ± 0.59	4.75 ± 0.59	4.24 ± 0.60	<0.001
10	entropy	4.87 ± 0.46	4.26 ± 0.59	4.79 ± 0.58	4.28 ± 0.60	<0.001

Table 3. Post hoc *p*-values between the phases of the protocol for differences in tone and entropy values with varying lags. P_{12} denotes post hoc *p*-value between the first baseline phase and heel stimulation phase. P_{13}, post hoc *p*-value between the first baseline phase and second baseline phase. P_{14}, post hoc *p*-value between the first baseline phase and heel stick blood drawing phase. P_{23}, post hoc *p*-value between the heel stimulation phase and second baseline phase. P_{24}, post hoc *p*-value between the heel stimulation phase and heel stick blood drawing phase. P_{34}, post hoc *p*-value between the second baseline phase and heel stick blood drawing phase.

	lag	P_{12}	P_{13}	P_{14}	P_{23}	P_{24}	P_{34}
tone	1	<0.001	>0.999	0.015	<0.001	0.052	0.013
	2	<0.001	>0.999	0.001	<0.001	0.008	0.001
	3	<0.001	>0.999	<0.001	<0.001	0.005	<0.001
	4	<0.001	>0.999	<0.001	<0.001	0.003	<0.001
	5	<0.001	>0.999	<0.001	<0.001	0.003	<0.001
	6	<0.001	>0.999	<0.001	<0.001	0.004	<0.001
	7	<0.001	>0.999	<0.001	<0.001	0.005	<0.001
	8	<0.001	>0.999	<0.001	<0.001	0.006	<0.001
	9	<0.001	>0.999	<0.001	<0.001	0.008	<0.001
	10	<0.001	>0.999	<0.001	<0.001	0.008	<0.001
entropy	1	0.002	0.990	0.023	0.005	0.863	0.055
	2	0.001	0.997	0.004	0.003	0.986	0.009
	3	<0.001	0.976	<0.001	<0.001	0.994	0.001
	4	<0.001	0.985	<0.001	<0.001	0.978	0.001
	5	<0.001	0.945	<0.001	<0.001	0.992	0.001
	6	<0.001	0.959	<0.001	<0.001	0.995	<0.001
	7	<0.001	0.931	<0.001	<0.001	0.987	<0.001
	8	<0.001	0.945	<0.001	<0.001	0.996	<0.001
	9	<0.001	0.932	<0.001	<0.001	0.99	<0.001
	10	<0.001	0.939	<0.001	<0.001	0.997	<0.001

(*p*-values: from lag 1 to lag 10 − <0.001), and Phase 3 and Phase 4 (*p*-values: lag 1 – 0.013, lag 2 – 0.001, from lag 3 to lag 10 – <0.001). Statistically significant differences were observed also between the two stress phases (Phase 2 and Phase 4, *p*-values: from lag 2 to lag 10 – <0.01), except at lag 1 ($p = 0.052$).

A similar pattern was observed comparing entropy values among Phases 1 and 2, Phase 1 and Phase 3, as well as Phase 3 and Phase 2. Phase 3 and 4 did not differ only at lag 1 ($p = 0.055$), while no significant differences were observed at any lag comparing Phases 2 and 4.

The results of ROC analyses are reported as AUC and 95% confidence intervals, between Phases 1 and 2, and Phases 1 and 4 (table 4 and figure 2). Statistically significant differences between the AUC values of tone and entropy, between Phases 1 and 2 were observed at every lag ($p < 0.05$), whereas

Table 4. Average AUC values with 95% confidence interval (CI) obtained using tone and entropy between the first baseline and the stress phases (heel stimulation and heel stick blood drawing). *P*-values were calculated using a two-tailed *t*-test and show the statistical significance of the difference in AUC values obtained using tone and entropy parameters. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 4, heel stick blood drawing phase.

	Phase 1 versus Phase 2					Phase 1 versus Phase 4				
	tone		entropy			tone		entropy		
lag	AUC	95% CI	AUC	95% CI	*p*-value	AUC	95% CI	AUC	95% CI	*p*-value
1	0.89	0.80–0.95	0.76	0.63–0.83	<0.000	0.74	0.62–0.82	0.69	0.54–0.80	0.026
2	0.88	0.74–0.95	0.76	0.67–0.87	<0.000	0.74	0.62–0.83	0.72	0.55–0.84	0.127
3	0.89	0.80–0.97	0.78	0.67–0.89	<0.000	0.77	0.68–0.86	0.75	0.62–0.83	0.242
4	0.89	0.70–0.95	0.77	0.63–0.88	0.002	0.78	0.63–0.85	0.76	0.65–0.90	0.450
5	0.89	0.74–0.95	0.76	0.65–0.87	0.002	0.79	0.68–0.89	0.78	0.66–0.90	0.355
6	0.88	0.78–0.97	0.77	0.65–0.88	0.001	0.78	0.66–0.86	0.78	0.68–0.87	0.394
7	0.88	0.72–0.95	0.78	0.60–0.88	0.003	0.80	0.69–0.87	0.78	0.68–0.88	0.393
8	0.87	0.74–0.94	0.77	0.61–0.84	0.021	0.79	0.68–0.88	0.79	0.67–0.86	0.290
9	0.87	0.75–0.95	0.77	0.63–0.88	0.005	0.79	0.64–0.87	0.69	0.54–0.80	0.347
10	0.87	0.72–0.93	0.77	0.58–0.85	0.006	0.79	0.69–0.86	0.72	0.55–0.84	0.375

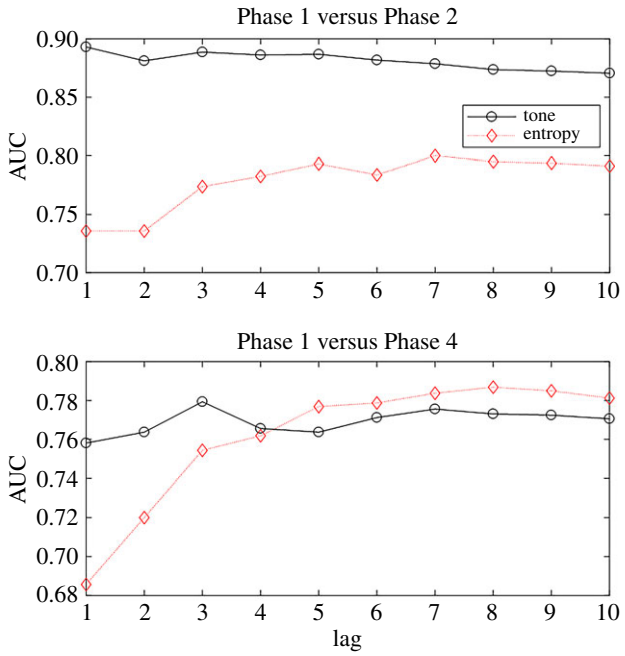

Figure 2. Average AUC value obtained using tone and entropy parameters for Phase 1 versus Phase 2 and Phase 1 versus Phase 4 with varying lag. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 4, heel stick blood drawing phase. (Online version in colour.)

comparing Phase 1 and 4, the AUC values of tone and entropy significantly differed only at lag 1 ($p = 0.026$). The AUC values of tone show consistency comparing Phase 1 and Phase 2, ranging from 0.87 at higher, to 0.89 at lower lags. The obtained AUC values comparing Phase 1 and 4 also showed consistency, ranging from 0.76 at lag 1, 2 and 5, up to 0.78 at lag 7.

In both cases, the discriminating performance of entropy increased with increasing lags, plateauing at lag 5. At higher lag values, entropy showed better performance than tone when comparing Phases 1 and 4. While comparing Phases 1 and 2, AUC values of tone were always greater.

AUC values obtained using both tone and entropy features at each lag using linear regression, showed consistency and high AUC values (AUC > 0.9) comparing Phases 1 and 2 (table 5 and figure 3). An increase of AUC with increasing lags was observed comparing Phases 1 and 4 performing best at lags 6 and 9 (AUC = 0.87). Significance testing showed better performance of the quadratic model discriminating between Phase 1 and 2, than Phase 1 and 4, at all lags.

4. Discussion

The multi-lag T–E method efficiently shows the changes in the ANS balance that can be used even with short time series. The physiological interpretation of both tone and entropy has been reported in different experimental settings [23,27,32,33]. A lower tone (negative values) indicates the parasympathetic predominance while a higher tone and lower entropy indicate a decrease in the parasympathetic and an increase or sympathetic predominance [23]. Although there is limited research on neonatal pain and stress applying HRV measures, this is the first study investigating multi-lag T–E in a neonatal stress framework [34,35].

It has been previously reported that healthy adults at rest have higher mean levels of entropy and lower and negative mean tone levels at different lags, showing a predominance in the parasympathetic branch of the ANS [22,23]. Our findings are comparable to that of adults, showing the same distribution of mean tone and entropy levels indicating a predominance of parasympathetic activity in term neonates when at rest [36,37].

Compared to the stress phases, at baseline, higher mean entropy values were observed than in the heel stick blood sampling phase, showing a reduction in randomness with increased sympathetic activity. Mean tone values are lower in the baseline phases compared with the stress phases, showing a predominance in parasympathetic activity

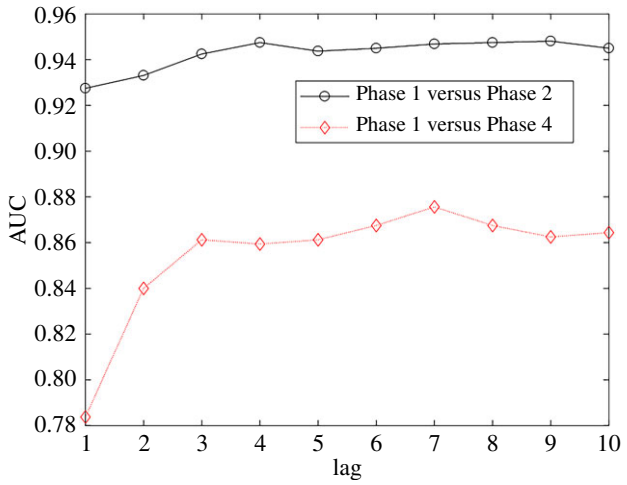

Figure 3. Average AUC value obtained using a linear regression model of both tone and entropy parameters for Phase 1 versus Phase 2 and Phase 1 versus Phase 4 with varying lag. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 4, heel stick blood drawing phase. (Online version in colour.)

Table 5. Average AUC values with 95% confidence interval (CI) obtained using both tone and entropy features at each lag using linear regression (quadratic model). *P*-values were calculated using a two-tailed *t*-test and show the statistical significance of the difference in AUC values obtained for 'Phase 1 versus Phase 2' and 'Phase 1 versus Phase 4' using both tone and entropy parameters. Phase 1, first baseline phase; Phase 2, heel stimulation phase; Phase 4, heel stick blood drawing phase.

	Phase 1 versus Phase 2		Phase 1 versus Phase 4		
lag	AUC	95% CI	AUC	95% CI	*p*-value
1	0.93	0.85–0.97	0.78	0.62–0.87	<0.001
2	0.93	0.85–0.97	0.84	0.72–0.92	0.001
3	0.94	0.85–0.97	0.86	0.76–0.93	0.001
4	0.95	0.89–0.98	0.86	0.74–0.92	0.001
5	0.94	0.86–0.97	0.86	0.77–0.94	0.001
6	0.95	0.87–0.98	0.87	0.73–0.93	0.001
7	0.95	0.87–0.99	0.88	0.75–0.94	<0.001
8	0.95	0.88–0.98	0.87	0.77–0.93	0.001
9	0.95	0.88–0.98	0.86	0.77–0.93	0.001
10	0.95	0.81–0.98	0.86	0.75–0.93	<0.001

during the baseline phase, and an increase in the sympathetic activity at both stress phases.

Statistically, when considering a time series, high entropy is related to information spread over many states, leading to difficult predictions, due to increased uncertainty or randomness. If we focus on the series of RR intervals, high entropy reflects a healthy responsive ANS. Conversely, a decrease in entropy during the stress phases indicates that the series of RR intervals is less erratic or less random. This is consistent with previous findings, that the HRV of neonates in stress phases results in: (1) an increasing part of mean-reversion effect compared to random fluctuations; (2) an increasing negative scaling exponent, depicting a higher autocorrelation of the accelerations in the RR_i series. This higher autocorrelation, acting as a fractional integral, smooths the series and thus reduces fluctuations [30,38].

The multi-lag part of our method is intended to take into account long-range influence of the baroreflex. This generalization of the T–E method to lags higher than one is consistent with many other methods that incorporate long-term effects. The particularity of the multi-lag T–E approach compared to fractal methods is that it determines the one lag that is the most significant, independent of any model relating various lags as is often stated in the fractal models. This model-free analysis of the lags therefore seems more general.

Another aim of this study was to estimate the diagnostic performances of tone and entropy and when combining both features. When separately comparing the resting and simulation phase, the performance of tone outperformed entropy, but a slight reversion of performance, especially at higher lags was shown when comparing the resting and blood sampling phase. When combined in a quadratic linear regression model, the AUC values increased in both cases. The lower performance comparing Phase 1 and 4 to Phase 1 and 2 might be related to the duration and type of stress that was applied. When sampling blood, pressure to the heel is routinely applied intermittently; after lancing, pressure to the heel is just enough to collect the required amount of blood to do further laboratory tests and then to stop the bleeding. In Phase 2, intermittent pressure only was applied to the neonates' heel for 90 s while at Phase 4, pressure was applied after lancing for blood collection and for haemostasis. These findings imply that multi-lag T–E may be useful for differentiating the type, duration and intensity of stress in the neonate.

As compared to mature nervous systems, the findings in stressed and resting term neonates match the distributions of tone and entropy to adults without and with cardiac autonomic neuropathy (CAN) [22,23,32]. It is well known that in patients with CAN due to the nerve damage, the parasympathetic activity is diminished and the sympathetic tone predominates [39]. These findings let us conclude that T–E can be used as a surrogate measure of ANS in neonates.

In another recent study, similar to ours though evaluating fetal HRV, the authors indirectly showed the evolution of the ANS [33]. Their results showed that tone increases and entropy decreases at all lags in the late-term fetuses, compared to fetuses with a younger estimated gestational age reflecting the maturation of the sympathetic nervous system as the fetus approaches the delivery period. Our study can be thought as an extension of their work, showing the changes in the ANS in healthy term neonates. The transition from intrauterine to extrauterine life is the most complex adaptation in human life [40]. Significant changes occur in the cardiovascular system during the first hours or perhaps first days of life to a transitional circulation that undergoes further development depending on the different circumstances of extrauterine life. Compared to our study, especially during the baseline phases, at all lags, tone is higher both in the early and late fetuses, while entropy was higher in our study group. Such findings might indicate a maturation both in the sympathetic and parasympathetic branch of the ANS, further showing that in our stress phases, a higher sympathetic response is observed. Although the researchers hypothesized that the maturation of the sympathetic branch might indicate preparation for delivery, transition in the circulation plays a role in ANS balance [40]. The higher dominance of the parasympathetic branch at rest might

imply that at the third day after delivery, the stress response caused during vaginal delivery has diminished.

The observed results of multi-lag T–E should not be limited only to scientific research, but rather they suggest a potential application the real-life NICU environment where vital parameters are routinely measured. Neonates admitted to the NICU are generally suffering from diseases for their specific age group, and may suffer from procedural pain varying from repeated venipunctures, umbilical catheter placements, tracheal suctioning, chest tube placements, lumbar punctures, etc. Owing to the applicability on short-term recordings, baseline values of multi-lag T–E can be easily obtained and applied to real-time measurements. A primer of such application may be useful in the titration of analgesics or sedation medicine, for longer lasting procedures. Similarly, an application of multi-lag T–E can be envisioned in the early onset of diseases with a subtle progression, i.e. necrotizing enterocolitis or perinatal infections, where earlier detection would provide opportunity for intervention or prevention.

In conclusion, we applied a novel, recently developed measure of HRV to a simple physiological model of acute neonatal stress. Multi-lag T–E provides new insight into the developing ANS and was able to distinguish resting from stressed neonates. The possibility to apply multi-lag T–E on short time segments makes it suitable as an additional tool for more objective assessment of neonatal stress.

Data accessibility. The data that support the findings of this study are available upon request from the corresponding author.

Authors' contributions. M.S. and C.K.K. contributed equally to this study. M.S., K.K. and K.M. designed the study, conducted the research and collected the data; M.S., K.K., K.M., M.P. and D.B. did the data preprocessing; C.K.K. developed the code required for analysis and analysed the data; K.K. carried out both intervention phases; J.Y., D.B., M.P. and P.D.A. interpreted the data, revised the draft and approved the final version; M.S., C.K.K., M.G. and P.D.A. wrote the first draft and final manuscript. All authors gave final approval for publication.

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