Validity of commonly used heart rate variability markers of autonomic nervous system function

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\textbf{Running head:} Validity of HRV markers

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Abstract

Background: Despite strong reservations regarding the validity of a number of heart rate variability measures, they are still being used in recent studies.

Aims: To compare the reactivity of ostensible sympathetic HRV markers (LF, VLF) to that of electrodermal activity, an exclusively sympathetic marker, in response to cognitive and orthostatic stress. To investigate the possibility of LF as a vagal-mediated marker of baroreflex modulation; and to compare the ability of HRV markers of parasympathetic function (RMSSD and HF) to quantify vagal reactivity to cognitive and orthostatic stress.

Results: None of the purported sympathetic HRV markers displayed a reactivity that correlated with electrodermal reactivity. LF (ms²) reactivity correlated with the reactivity of both RMSSD and HF during baroreflex modulation. RMSSD and HF indexed the reactivity of the parasympathetic nervous system under conditions of normal breathing, however, RMSSD performed better as a marker of vagal activity when the task required breathing changes.

Conclusions: Neither LF (ms²), LF (nu), nor VLF represent cardiac sympathetic modulation of the heart. LF (ms²) may reflect vagally-mediated baroreflex cardiac effects. HRV linear analysis therefore appears to be restricted to the determination of vagal influences on HR. With regards to HRV parasympathetic markers, this study supports the suggestion that HRV frequency domain analyses, such as HF, should not be used as an index of vagal activity in study tasks where verbal responses are required as they may induce respiratory changes great enough to distort HF power.

Keywords: heart rate variability, autonomic reactivity, stress, LF, VLF, electrodermal activity
Introduction

Heart rate variability

Heart rate variability (HRV) measures the oscillations in the interval between consecutive heartbeats, as well as the oscillations between consecutive instantaneous heart rates [1]. These oscillations result from complex, non-linear interactions and HRV is thus considered a measure of neuro-cardiac function that represents heart-brain interactions, as well as autonomic nervous system dynamics [2]. An optimal level of variability in the heart rate signal is critical to the flexibility and resilience that characterizes health. While too much instability is detrimental to efficient physiological functioning, too little variation similarly indicates pathology [2].

HRV analysis

HRV is measured using electrocardiogram or photoplethysmograph sensors that detect the cardiac interbeat interval (IBI). Currently, various analyses of the resultant IBI signal exist; each with their own advantages, challenges and limitations. Current analyses can be divided into linear algorithms, including time-domain and frequency-domain indices, and non-linear algorithms, such as the Poincaré plot and entropy-based analyses.

Time-domain analyses

Time domain analyses calculate either the heart rate at any point in time or the intervals between successive normal QRS complexes [1]. The time-domain measures most often employed in HRV research appear to be the standard deviation of all NN intervals (SDNN) and the square root of the mean of the sum of the squares of differences between adjacent NN intervals or root mean square of successive differences (RMSSD). SDNN is used as an estimate of overall HRV, while RMSSD represents an estimate of vagal or parasympathetic activity.

Frequency-domain analyses

Power spectral density analysis can be used to separate the IBI series into the component rhythms operating within different frequency ranges. The main advantage of spectral density analysis is that it supplies frequency and amplitude information about specific rhythms existing in the HRV waveform, and therefore provides a means to quantify the various oscillations [2].

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Values are expressed as the power spectral density (in ms$^2$), which is the area under the curve in a given segment of the spectrum. Three main spectral components are distinguished in an HRV spectrum calculated from short-term recordings: high frequency (HF), low frequency (LF) and very low frequency (VLF) bands. The HF band represents the power in the frequency range between 0.15 and 0.4 Hz, while the LF band ranges from 0.04 to 0.15 Hz, and the VLF band represents the power below 0.04 Hz [1].

With at least one exception [3], HF power is generally believed to represent respiratory-linked changes in heart rate and is therefore widely accepted as a measure of respiratory sinus arrhythmia (RSA), or the parasympathetic contribution to HRV. RSA refers to the acceleration in heart rate that occurs during inspiration (due to the cardiovascular control centre’s inhibition of vagal outflow) and the subsequent heart rate deceleration that occurs during expiration (due to vagal restoration) [2]. The stronger these variations in heart rate, the larger the RSA and the stronger the vagal cardiac control [4]. HF power is generally, but not under all conditions, correlated with the previously mentioned time domain measure RMSSD [5].

The origin of LF power, however, has provoked consistent controversy. LF power has, for long, been considered a measure of sympathetic activity, with a contribution by the parasympathetic nervous system [1] and is in many recent HRV studies still considered as such [6–9]. Some even utilize LF as a putative marker of pure sympathetic function [10]. However, uncertainties regarding the ability of the LF marker to accurately measure the activity of the sympathetic nervous system have consistently been expressed since the 1990’s. In fact, the 1997 review by Eckberg [11] eloquently summarized the evidence against the use of LF as a sympathetic marker and suggested that the notion that LF can track changes in sympathetic nerve activity should be refuted. This viewpoint has since received support [12–15]. In fact, evidence that strongly contradicts the assumption of LF as a sympathetic marker, and suggests that LF activity is instead determined by the parasympathetic nervous system, has been eloquently summarized in a recent review by Reyes del Paso et al [16]. Whatever the origin of the LF frequency band, it appears to be generally accepted in the HRV literature that LF power (expressed in absolute units or ms$^2$) does not reflect pure sympathetic activity.

In order to quantify HRV as a reliable marker of cardiac ANS control, some authors recommended the use of normalized units (nu) when reporting power spectral density. This viewpoint stems from studies that have shown that conditions associated with an increase in sympathetic activity produce a decrease in overall HRV power, whereas the opposite occurs
during vagal activation. These authors contend that when spectral components are expressed in absolute units (ms$^2$), the true estimation of LF and HF power is distorted by changes in total spectral power [17,18]. Normalized units are obtained by dividing the power of the given component (LF or HF) by the total power minus the VLF power. Based on the fact that normalized units are determined by a re-computation of LF power that relates it to overall HRV, older studies have suggested that the LF component, when expressed in normalized units, represents a measure of pure sympathetic activity [19–21]. Despite evidence to the contrary, incidences of the use of LF (nu) as a marker of sympathetic activity can still be seen in recent HRV literature [9,22–27]. In line with this thought, the ratio of LF (nu) to HF (nu) was originally suggested to represent the sympathovagal balance, with an increase in the LF/HF ratio indicating a predominance of sympathetic activity and a decrease in the LF/HF ratio indicating parasympathetic predominance. This view has been highly criticized due to the controversial and uncertain relationship between LF power and sympathetic activity and the non-reciprocal and non-linear relationship between sympathetic and parasympathetic activity [5,11,15]. However, a number of recently published studies still rely on the use of the LF/HF ratio as an indicator of sympathovagal balance [7,8,22–24,27–29]. In fact, a recent review by Laborde et al [5] indicated that 65% of HRV studies are still basing their conclusions on the LF/HF ratio. Some authors even suggest that calculation of the LF/HF ratio removes the parasympathetic or vagal contribution to HRV and leaves a more refined indication of purely sympathetic function [30].

The guidelines from the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [1] suggested VLF to be a dubious measure that should be avoided. However, a relatively recent study has suggested that the VLF rhythm is intrinsically generated by the heart and that the amplitude and frequency of these oscillations are modulated by efferent sympathetic activity [2]. Therefore, some studies have actually utilized VLF power as a reflection of sympathetic tone [2,31–34].

**Electrodermal activity**

Electrodermal activity is a non-invasive peripheral marker of sympathetic nervous system activity that is widely used in psychophysiological research [35]. The eccrine sweat glands are exclusively controlled by sympathetic sudomotor cholinergic neurons, with no associated parasympathetic innervation. The activity of the eccrine sweat glands is reflected in the conductance of an applied current at the surface of the skin, termed skin conductivity or
electrodermal activity. When sweat glands are activated, they produce sweat that is passed to the stratum corneum of the epidermis, allowing the corneum to become conductive. An increase in skin conductance is thus considered proportional to sweat gland activation and sweat secretion.

Reactivity to stressors

Central Autonomic Network

A central autonomic network (CAN), which includes reciprocally interconnected areas of the prefrontal cortex (PFC), limbic system and brainstem, has recently been identified in humans. The CAN forms a critical component of an internal regulatory system through which the brain controls visceromotor, neuroendocrine and behavioural responses critical for goal-directed behaviour, adaptability and health [36–38]. The CAN is believed to generate context-appropriate responses and regulate psychophysiological resources related to goal-directed behaviour via modulation of the PFC [36]. Neuroimaging studies suggest the core of the CAN consists of the left amygdala, right anterior and left posterior insula, and midcingulate cortices [39]. The PFC, specifically the insula and cingulate cortices, exerts tonic inhibitory control on the sympathoexcitatory subcortical threat circuits [36,40] and tonically inhibits the amygdala via pathways leading to intercalated gamma-aminobutyric acid (GABA)-ergic amygdala neurons [40]. During uncertainty, novelty or threat the PFC becomes hypoactive, allowing the central nucleus of the amygdala, and the corresponding sympathoexcitatory circuits to become disinhibited [36,40]. When the PFC is taken offline it allows automatic, predominantly non-voluntary, behaviours associated with neural structures like the amygdala to dominate without delay [40]. Effects of this disinhibition are apparent in organs controlled by the autonomic nervous system, such as the heart and eccrine sweat glands, allowing for the calibration of organ responses with contextually appropriate and adaptive behaviours. The central electrodermal regulation system has been shown to consist of areas that form part of the CAN and electrodermal responses can be elicited by direct electrical stimulation of areas of the CAN, such as the cingulate, insula and amygdala [41]. Furthermore, lesions to some of these areas have been shown to reduce the magnitude of EDA responses [41]. These findings therefore propose a correspondence in the neuroanatomical areas that support bodily arousal through the sympathetic nervous system and attentional processes, suggesting that electrodermal activity can be used as an index of attention [41]. Similarly, output of the CAN is also believed to be mediated via pre- and post-ganglionic autonomic neurons of the stellate ganglia and vagus
nerve that innervates the sinoatrial node of the heart [42]; and the output of the CAN is therefore believed to be directly linked to HRV [36,38]. Moreover, all central levels of EDA regulation are known to also be involved in the neural control of heart rate [43].

Stroop test as cognitive stressor
The Stroop test, originally introduced by John Ridley Stroop in 1935, requires participants to name the colour in which a word is printed or appears on a computer screen, ignoring the word itself. In the congruent condition of the task, the stimulus word matches the stimulus colour (e.g., BLUE written in blue ink) and participants rely on well-learned reading processes to produce both fast and accurate responses. In the incongruent condition (e.g., BLUE in red ink), accurate responding requires participants to use cognitive control mechanisms to inhibit word reading and activate colour-naming processes [44]. The performance cost in the incongruent condition is called the Stroop effect or Stroop interference and the incongruent condition is therefore regarded as a task that taxes executive function [45] and thus induces cognitive stress.

Different tasks or stressors are associated with disparate autonomic regions within the CAN, with cognitive tasks predominately associated with sympathetic regions [39]. Cognitive tasks, such as the Stroop test, are believed to increase activation of the sympathetic outflow to various systems in order to allow the organism to deal with the cognitive stress. Fechir et al [46], for instance, showed that the Stroop test was able to globally activate the SNS, resulting in changes in heart rate, pulse wave transit time, electrodermal activity, electromyography of the trapezius muscle and peripheral vasoconstriction, while Mestanik et al [47] showed that the Stroop test increases both alpha-adrenergic (vascular) and beta-adrenergic (cardiac) sympathetic activity. Indeed, the anterior cingulate cortex (ACC), which is an area that has been identified as a key regulatory area of the CAN [39], has been shown to be maximally activated during Stroop performance and disruption in its activity reduces the Stroop effect [47]. Since generalized sympathetic activation is believed to occur during Stroop performance, it is plausible to assume that the activation of different sympathetic subcomponents (such as the heart and eccrine sweat glands) should correspond during the application of this stressor.

Orthostatic stress
Upon standing up from the supine position, the effect of gravity causes blood to accumulate in the lower extremities, resulting in a decrease in thoracic venous blood volume and central
venous pressure. This causes a drop in venous return, which, through its negative effect on stroke volume, causes mean arterial blood pressure to fall. The decrease in mean arterial pressure decreases the firing rate of arterial baroreceptors, integrated in the cardiovascular control centre of the medulla oblongata, resulting in the stimulation of vasomotor and cardioacceleratory (i.e. sympathetic) centres, and an inhibition of cardioinhibitory (i.e. parasympathetic) centres. This baroreceptor reflex occurs in order to restore the mean arterial pressure.

**Aim**
The present study aimed to assess the ability of universally used HRV markers of parasympathetic function (i.e. HF and RMSSD) to quantify vagal activity. Moreover, the study aimed to determine whether the reactivity of any of the purported sympathetic HRV measures (i.e. LF (ms²), LF (nu) and VLF) correlated with the reactivity of an accepted marker of sympathetic activity (electrodermal activity) during generalized sympathetic activation. Although, as discussed in the introduction, doubts have previously been raised regarding the ability of these markers to represent sympathetic activity, this study undertakes to clarify some of the controversy by utilizing a novel approach in which the activity of these markers during sympathetic activation are compared to that of a well-known sympathetic marker. In order to elucidate the origin of the LF power component, the study further aimed to determine whether the reactivity of LF correlated with the reactivity of universally used HRV markers of parasympathetic activity (RMSSD and HF) during a task that involves baroreflex modulation in the form of orthostatic stress.

**Materials and Methods**

**Sample**
The study protocol was approved by the University of Johannesburg’s Faculty of Health Sciences Research Ethics Committee (approval number AEC01-31-2014), as well as the University of Pretoria’s Faculty of Health Sciences Research Ethics Committee (339/2014), and was conducted in accordance with the Declaration of Helsinki guidelines. The sample comprised of fifty seven first year students in the Faculty of Health Sciences at the University of Johannesburg. Signed informed consent was obtained from all participants and participants were free to withdraw from the study at any time. Students were excluded from the study if
they had been diagnosed with any cardiovascular disease or disorder or if they were taking any medication that is known to have an effect on the cardiovascular system.

**Heart rate variability**

Heart rate variability was determined by analysis of the IBIs obtained by means of the Actiheart heart rate monitoring device from CamNtech (Cambridge, UK). The IBIs were analysed using Kubios HRV Analysis Software 2.2 developed by The Biosignal Analysis and Medical Imaging Group from the University of Eastern Finland [48]. Frequency components were calculated using Fast Fourier Transformation (FFT). Before FFT was employed all imported data was de-trended, using the smoothness priors method [49] to remove any disturbing low frequency baseline trend components, and then interpolated at a sampling rate of 4 Hz. Artifact correction of a medium filter power was applied to the raw tachogram. In order to determine the reactivity of the HRV measures to the applied stressors changes in HRV values (∆ values) were calculated (∆cognitive = Stroop test HRV value - baseline HRV value; ∆orthostatic = standing HRV value - supine HRV value).

**Electrodermal activity**

The EDA signal was collected through two MLT117F silver/silver chloride electrodes attached by adhesive collars to the palmar surface of the middle and index fingers on the participants’ left hand. Participants’ fingers were cleaned prior to sampling to ensure the removal of surface salt. The mean EDA signal, in µSiemens, was calculated using LabChart 8 software (version 8.1.4) from AD Instruments (Sydney, Australia). In order to determine the reactivity of the EDA signal to the applied stressors, changes in EDA values (∆ values) were determined (∆cognitive = Stroop test EDA value - baseline EDA value; ∆orthostatic = standing EDA value - supine EDA value).

**Procedure**

Participants were asked to refrain from exercise or alcohol consumption for 24 hours prior to testing. They were also asked to refrain from smoking for four hours prior to testing, since nicotine has been shown to exhibit an elimination half-life of two hours [50]. Moderate food consumption was allowed, however, participants were asked to refrain from consuming coffee, tea or caffeine-containing soft drinks for two hours prior to testing. Participants arrived at the laboratory and were fitted with the Actiheart heart rate monitor and the electrodermal
After the equipment was fitted, baseline recordings were performed for 10 minutes, during which the participants were asked to sit quietly in a quiet environment at a room temperature of approximately 20-22°C. Only the final five minutes of the baseline recording was used for the analysis, in order to allow for the stabilization of ANS parameters and acclimatization to the recording environment. After the baseline recording, participants performed the incongruent condition of the Stroop test on the ML4818 PowerLab 15T system from AD Instruments for five minutes in order to assess their response to a cognitive stressor. The version of the Stroop test used in this study utilized five colours (green, blue, red, purple and brown) and required participants to verbally report their answers. Therefore no button presses were required. Three trials of 120 words were available, however participants were asked to complete as many words as possible during the 5 minute period. To test their response to an orthostatic stressor, participants were asked to lie in a supine position for approximately 10 minutes and then asked to stand upright, with their feet approximately 30 centimetres apart, and their backs 30 centimetres away from the wall, for five minutes. Once again, only the final five minutes of the supine recording was used for the analysis in order to allow for the stabilization of ANS parameters. As discussed later, no controlled or paced breathing was implemented during any of the conditions.

**Statistical analyses**

Since the data did not show a normal distribution, a Wilcoxon signed-rank test was used in order to determine whether any statistically significant reactivity of the markers occurred in response to the applied stressors. In order to determine the correlation between electrodermal reactivity and ostensible sympathetic HRV marker reactivity to the applied stressors, Spearman's rank correlation coefficients were used. Due to the lack of any significant correlations between the examined variables, the data was further investigated with Cohen's kappa coefficient, in order to determine the extent of the inter-rater agreement between the markers. Spearman's rank correlation coefficients were used in order to determine the correlation between LF and parasympathetic HRV reactivity in response to the orthostatic stressor. A $p$ value < 0.05 was considered significant for all statistical analyses.
Results
The mean age of the sample was 19.89 years (SD = 2.64). The sample was predominantly female (n=46/57; 81%). Ethnicity distribution was as follows: Black (n=35/57; 61%); White (n=16/57; 28%); Indian (n=5/57; 9%); Coloured (n=1/57; 2%).

Reactivity to applied stressors
As seen in Table 1, and as expected, a statistically significant increase in electrodermal activity ($p < 0.001$) was observed in response to the Stroop test. Furthermore, a statistically significant increase in electrodermal activity ($p < 0.001$) was also observed in response to orthostatic stress. With regards to the purported sympathetic HRV markers, only LF (nu) ($p = 0.003$) significantly increased in response to the Stroop test, and only LF (nu) ($p < 0.001$) showed a statistically significant increase in response to orthostatic stress. With regards to the parasympathetic HRV markers, RMSSD ($p < 0.001$) and HF (ms$^2$) ($p < 0.001$) both significantly decreased in response to orthostatic stress, reflecting the well-known vagal withdrawal that occurs during the shift from supine to standing. Although this same shift in RMSSD ($p < 0.001$) occurred in response to the Stroop test, there was no statistically significant decrease in HF (ms$^2$) ($p = 0.26$).
### Table 1. Autonomic nervous system marker reactivity in response to cognitive and orthostatic stress

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cognitive stress</th>
<th>Orthostatic stress</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean change from baseline</td>
<td>SD</td>
<td>95% CI</td>
<td>p value&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔEDA (µS)</td>
<td>6.15</td>
<td>3.98</td>
<td>(5.09; 7.20)</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔLF (ms²)</td>
<td>284.02</td>
<td>2319.46</td>
<td>(-331.42; 899.45)</td>
<td>0.08</td>
</tr>
<tr>
<td>ΔHF (ms²)</td>
<td>9.97</td>
<td>22.02</td>
<td>(4.12; 15.81)</td>
<td>0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔVLF (ms²)</td>
<td>9.00</td>
<td>322.04</td>
<td>(-76.45; 94.45)</td>
<td>0.95</td>
</tr>
<tr>
<td>ΔRMSSD (msec)</td>
<td>-10.02</td>
<td>18.28</td>
<td>(-14.86; -5.17)</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔHF (ms²)</td>
<td>-177.28</td>
<td>1485.11</td>
<td>(-571.33; 216.77)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

EDA = electrodermal activity; LF = low frequency; VLF = very low frequency; RMSSD = root mean square of successive differences; HF = high frequency

<sup>a</sup> Wilcoxon signed-rank test

<sup>b</sup> p < 0.01
Correlation between EDA and ostensible sympathetic HRV marker reactivity

As indicated in Table 2, almost none of the HRV values purportedly believed to represent the activity of the sympathetic nervous system displayed a reactivity (Δ values) that correlated with electrodermal reactivity (Δ values), in response to either the Stroop test or orthostatic stress. The only statistically significant correlation found was between electrodermal reactivity and the reactivity of VLF ($r_s = 0.29$, $p = 0.03$), however this relatively small correlation was only found in response to the Stroop test and not orthostatic stress.

Table 2. Correlation between EDA reactivity and reactivity of ostensible sympathetic HRV markers (Δ values)

<table>
<thead>
<tr>
<th>Correlates</th>
<th>Cognitive stress</th>
<th>Orthostatic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$ $p$ value</td>
<td>$r_s$ $p$ value</td>
</tr>
<tr>
<td>EDA (µS): LF (ms$^2$)</td>
<td>0.07 0.62</td>
<td>-0.15 0.26</td>
</tr>
<tr>
<td>EDA (µS): LF (nu)</td>
<td>0.05 0.72</td>
<td>0.18 0.18</td>
</tr>
<tr>
<td>EDA (µS): VLF (ms$^2$)</td>
<td>0.29 0.03$^a$</td>
<td>-0.15 0.26</td>
</tr>
</tbody>
</table>

EDA = electrodermal activity; LF = low frequency; VLF = very low frequency

$p < 0.05$

Correlation between LF and PSNS HRV marker reactivity

As indicated in Table 3, no statistically significant correlation between LF (ms$^2$) reactivity and the reactivity of RMSSD and HF (ms$^2$) was found in response to cognitive stress. However, LF (ms$^2$) reactivity positively correlated with the reactivity of both RMSSD ($r_s = 0.67$; $p < 0.001$) and HF (ms$^2$) ($r_s = 0.68$; $p < 0.001$) in response to orthostatic stress. As expected, LF (nu) reactivity correlated negatively with the reactivity of both RMSSD and HF (ms$^2$) in response to both cognitive ($r_s = -0.34$; $p = 0.01$ and $r_s = -0.45$; $p < 0.001$, respectively) and orthostatic stress ($r_s = -0.33$; $p = 0.01$ and $r_s = -0.30$; $p = 0.02$, respectively).
Table 3. Correlation between ostensible sympathetic marker reactivity and reactivity of parasympathetic HRV markers (Δ values)

<table>
<thead>
<tr>
<th>Correlates</th>
<th>Cognitive stress</th>
<th>Orthostatic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p value</td>
</tr>
<tr>
<td>LF (ms²) : RMSSD (msec)</td>
<td>0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>LF (ms²) : HF (ms²)</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>LF (nu) : RMSSD (msec)</td>
<td>-0.34</td>
<td>0.01ᵃ</td>
</tr>
<tr>
<td>LF (nu) : HF (ms²)</td>
<td>-0.45</td>
<td>&lt;0.001ᵇ</td>
</tr>
</tbody>
</table>

LF = low frequency; RMSSD = root mean square of successive differences; HF = high frequency

¹p < 0.05
²p < 0.01

Discussion

HRV analysis is, with some methodological issues, accepted by most as a reliable method for the assessment of parasympathetic function. However, ambiguity exists with regards to the evaluation of sympathetic activity.

Parasympathetic HRV markers

Respiratory influence on HRV

Controlling for respiration has been a long and ongoing debate within HRV research. The reasoning behind this debate is that, in certain conditions, HRV can be affected by respiratory depth and frequency, as well as the central respiratory drive [5]. Therefore, some studies have suggested that RSA (and therefore RSA-based HRV measures) lack the ability to track vagal fluctuations under normal non-paced breathing [51]. In contrast, others contend that pacing of breathing and statistical adjustments of pulmonary variables actually distort true RSA, since it represents a naturally occurring rhythm in the heart rate pattern at approximately the frequency of spontaneous breathing [5,52]. In fact, a substantial body of evidence from behavioural genetic, neuroimaging, cardiorespiratory coupling, and psychophysiological studies suggests that by removing the variance associated with respiration from HRV, one would inadvertently remove the variance associated with the common neural origin of HRV and respiration (i.e. the variance that the HRV researcher is actually interested in) [5]. Furthermore, it appears that the effect of respiratory depth and frequency on parasympathetic HRV measures is generally not substantial and uncorrected RSA, i.e., RSA measured under conditions of non-paced breathing.
is sufficient to index within-participant changes in cardiac vagal activity in studies which do not involve major changes in respiration [53]. In support of this viewpoint, some authors have re-computed analyses involving RSA by controlling for respiratory frequency and have shown that in no cases did this re-computation eliminate the reported effects [4,53,54]. It is thus believed that the relationship between inter- and intra-individual changes in respiratory parameters and vagal activity is not improved by controlling for respiration, and that spontaneous breathing is best when experimental tasks only elicit moderate changes in respiration [5,55].

The question that therefore remains is what is meant by moderate changes in respiration. It is believed that when breathing remains between nine cycles per minute (0.15 Hz) and 24 cycles per minute (0.40 Hz), HRV measures accurately reflect vagal activity [5]. Therefore, as indicated in the review by Laborde et al [5], it is useful to have some indication of the respiratory rate of each of the participants involved in the study, in order to confirm that the participants were breathing at a “normal” rate. An estimate of respiratory rate can be derived from the central frequency of the HF component detected in an autoregressive analysis of heart rate, since the central frequency of HF has been shown to correlate significantly with strain gage measures of respiration [5]. A limitation of this method, however, is that there should be an observable HF peak or else it is questionable whether any true HF power exists [5].

Although HF does appear to be influenced by respiratory rates and depths under certain conditions, the time-domain measure RMSSD appears to be relatively free of respiratory influences [5,56]. Therefore, as recommended by Larborde et al [5], it is important to always couple the HRV frequency analysis with time-domain parameters (such as RMSSD) that index vagal activity. We therefore included both HF and RMSSD as indices of parasympathetic activity in this study.

Vagal reactivity to applied stressors
In line with majority evidence, recordings in the present study were done under conditions of non-paced breathing. Inspection of our data revealed that all participants had a central HF frequency between 0.15 and 0.40 Hz during autoregressive analysis of heart rate, both at baseline and during the application of cognitive and orthostatic stress. However, some of our participants did not have an observable HF peak during cognitive stress which, as discussed below, could be attributed to the fact that the participants had to verbally report their responses
during the Stroop test, and therefore may have experienced speech-related influences on their respiratory patterns.

As can be seen in Table 1, both RMSSD and HF (ms\(^2\)) showed statistically significant decreases in response to orthostatic stress, highlighting the well-known vagal withdrawal that occurs during the shift from supine to standing. Furthermore, the reactivity of these two markers correlated significantly during orthostatic stress (\(r_s = 0.91\); \(p < 0.001\)), suggesting that both RMSSD and HF (ms\(^2\)) adequately reflected the response of the parasympathetic nervous system to orthostatic stress. This decrease in RMSSD in response to a shift from supine to standing has been found in previous studies [57,58], while the decrease in HF (ms\(^2\)) in response to standing is also well documented [57–66].

With regards to the reactivity to cognitive stress, RMSSD showed a statistically significant decrease during the Stroop test, emphasizing the expected vagal withdrawal that occurs in response to this cognitive stressor. This finding has also been shown in previous studies [67,68]. However, HF (ms\(^2\)) activity did not significantly differ between baseline and application of cognitive stress. Furthermore, the reactivity of RMSSD and HF (ms\(^2\)), although correlated (\(r_s = 0.69\); \(p < 0.001\)), did not correlate to the same extent as was seen during the reactivity to orthostatic stress. The most plausible explanation for this finding is that participants were asked to report their responses on the Stroop test verbally, at their own pace. Therefore, the intentional breathing changes that occurred during talking may have affected the HF marker results in some participants, assumedly those who maintained a high pace. This conclusion is reinforced when the results of studies where verbal responses in the Stroop test were required are compared to the results of studies where no verbal responses were necessary. A significant decrease in HF (ms\(^2\)) in response to the Stroop test was, for instance, found in previous studies where the participants did not have to report their responses verbally [63,67,68], while a lack of any statistically significant change in HF (ms\(^2\)) in response to the Stroop test was found in a study by Garafova et al [69], where participants were asked to verbally report their responses. Therefore, both RMSSD and HF (ms\(^2\)) do appear to be able to index the activity of the parasympathetic nervous system; however, when a study task may induce substantial respiratory changes, then both variables should be included as markers of parasympathetic activity.
Sympathetic nervous system assessment

In order to examine the validity of purported HRV markers of sympathetic function, their results have to be comparable to that obtained by generally accepted methods. The two physiological measures that, at present, appear to present the least ambiguity are cardiac pre-ejection period and electrodermal activity.

Cardiac pre-ejection period is a marker commonly used in psychophysiological research as a measure of sympathetic cardiac modulation, as it reflects changes in left ventricle contractility [53]. However, cardiac pre-ejection period is known to be affected by cardiac preload and afterload, and is dependent on the amount of circulating adrenaline and the affinity and expression of left ventricular adrenoceptors, which are believed to change rapidly in response to different conditions, such as exercise and mental stress [53]. Therefore, using cardiac pre-ejection period as a measure of sympathetic cardiac modulation when comparing different conditions or tasks raises concerns.

Electrodermal activity as a marker of sympathetic nervous system reactivity

The complex central regulatory system of electrodermal activity has not yet been fully elucidated, but areas that are believed to be involved in central control of EDA include PFC areas, the amygdala and the anterior cingulate cortex [70]. These areas have been shown to be part of the CAN and therefore the effect of stressors that are able to activate the CAN, and thereby result in global sympathetic activation, can be quantified via electrodermal activity.

In the present study, measurement of electrodermal activity was performed under temperature controlled conditions which should have eliminated a possible influence by environmental temperature fluctuations. Consequently, a statistically significant increase in electrodermal activity ($p < 0.001$) was observed in response to the Stroop test. The statistically significant increase in electrodermal activity observed in response to the Stroop test is in line with the expected activation of the sympathetic nervous system that is known to occur in response to this stressor. This result is also in agreement with previous findings of an increase in electrodermal activity in response to the Stroop test in various study populations [35,71–78]. The present study also found a statistically significant increase in electrodermal activity in response to orthostatic stress ($p < 0.001$). Similar results have been found by previous studies where the change in position from supine to standing was shown to be associated with a statistically significant increase in skin conductivity [59,73,76,79]. These findings suggest that
a more generalized sympathetic response occurs during the adaptation to standing, and that it is not just the heart and blood vessels that are specifically activated during the reaction to orthostatic stress.

Although the odd remark has been noted about the fact that electrodermal activity represents the cholinergic (and not adrenergic) part of the sympathetic nervous system, it should be noted that, but for the difference at the neuro-effector junction, the tract conforms to that of the typical sympathetic effector pathway. Interestingly, a study has shown that, among a number of other physiological signals, heart rate and skin conductance provided the highest correlation with subjective stress levels in drivers, reaching an accuracy of over 97% [80].

These findings emphasize the utility of electrodermal activity as an accurate measure of sympathetic activity and therefore confirm that electrodermal activity can indeed be used as a marker of sympathetic activity in studies of autonomic function.

Ostensible sympathetic HRV markers

Reactivity to applied stressors

A major controversy that exists within HRV research is the question of whether linear HRV variables can, in any way, reflect sympathetic activity.

In the present study, two of the ostensible linear sympathetic markers of HRV did not show any statistically significant reactivity to either cognitive or orthostatic stress (see Table 1). This on its own questions their ability to accurately represent the activity of the sympathetic nervous system, as both stressors were shown to cause sympathetic activation in the present study, as well as in previous studies described in an earlier paragraph [35,59,71–73,76–78]. The only purported sympathetic HRV marker that showed a statistically significant increase in response to both stressors was the LF spectral component when expressed in normalized units, i.e., as LF (nu). Statistically significant increases in LF power, when expressed in normalized units, have been reported in many previous studies for both the Stroop test [68] and head-up tilt [60–65,76,81,82]. With regards to the absolute units of LF, a small number of studies have reported a significant increase in LF (ms²) in response to orthostatic stress [62,66,76]. However, many studies found no statistically significant change [57,64,65,79,83,84] and some even found a decrease in LF, when expressed in absolute units, in response to orthostatic stress [58,60,63,85]. Therefore our results appear to agree with the majority of findings where
significant increases in LF (nu) were found in response to both stressors and no statistically significant change in LF (ms²) in response to either stressor was observed (see Table 1).

Although LF (nu) did significantly increase in response to both stressors, the lack of correlation between LF (nu) and EDA reactivity casts doubt on the ability of LF (nu) to accurately represent the activity of the sympathetic nervous system. In addition, several issues render the scientific correctness of the normalization process contentious, as normalization of the power components is based on the physiological assumption of autonomic reciprocity. Autonomic reciprocity suggests that the sympathetic and parasympathetic branches of the autonomic nervous system are subject to reciprocal central control in the sense that increased activation of one system is always accompanied by inhibition in the other [16]. Cardiac autonomic reciprocity is not supported by current research and the two ANS branches have been shown to interact in a dynamic fashion, with either reciprocity or co-activation of both branches occurring, suggesting that the use of normalized units is indeed rather questionable [16]. Furthermore, the computation of normalized units is limited to the attainment of a ratio between LF and HF parameters and can therefore lead to the production of artificial statistically significant findings [16]. In order to express the frequency as normalized units, the power of the given component (LF or HF) is divided by the total power minus the VLF power. In the case of LF, LF (nu) is therefore equal to LF/(TP-VLF), which can also be expressed as LF (nu) = LF/(LF+HF). By building HF into the equation, artificially significant findings between LF (nu) and other variables might be found due to the correlation between those said variables and HF. This flaw in the normalization process was highlighted in our study with regards to marker reactivity in response to orthostatic stress. As can be seen in Table 1, LF (ms²) did not show a statistically significant shift in response to the application of orthostatic stress (p = 0.50), however, HF (ms²) did (p < 0.001). By building HF into the equation, the normalization process produces an artificial statistically significant shift in LF (nu) in response to orthostatic stress (p < 0.001). This flaw in the normalization process is also highlighted in Table 3 where LF (ms²) reactivity did not significantly correlate with parasympathetic reactivity in response to cognitive stress, however, by building HF inversely into the normalization equation, LF (nu) shows a statistically significant negative correlation with HF (ms²), once again resulting in the production of artificially significant correlations. Another issue associated with the normalization process becomes apparent in Table 3 when one looks at the response to orthostatic stress. LF (ms²) reactivity positively correlated with both RMSSD and HF (ms²) in response to standing, however, due to the equation used to normalize LF power, and the fact
that HF is inversely built into the equation, LF (nu) shows statistically significant negative correlations with both RMSSD and HF reactivity. Therefore, besides resulting in the production of artificial statistically significant findings, the normalization process may also distort true statistically significant findings.

**Correlation with electrodermal activity (EDA)**

As previously discussed, studies have shown that the Stroop test is able to induce generalized sympathetic activation [46,47]. Furthermore, disorders where augmented sympathetic modulation is indicated, such as schizophrenia, have shown augmented coupling between heart rate and skin conductance [43], while sympathetic HR-based markers that are believed to represent sympathetic activity to some extent (i.e. QT variability index (QTvi), as well as the ratio of approximate entropy of the QT interval to the approximate entropy of the of R-R interval (ApEnQT/ApEnRR)), have been shown to correlate with electrodermal activity during exercise [86]. Similarly, phasic electrodermal activity and heart rate accelerations have been shown to exhibit interdependent changes under relatively spontaneously conditions in middle-aged men, suggestive of a common central regulation and organization for both [87]. In the present study the reactivity of the ostensible sympathetic markers did not correlate with electrodermal reactivity, supporting the misgivings about their validity as sympathetic markers.

One may perhaps argue that interactive effects of cardiac sympathetic and parasympathetic neurons (i.e. accentuated antagonism effects) might result in a lack of correlation between HRV measures and electrodermal activity, which has no corresponding parasympathetic influence [53]. However, it is important to note here that previous studies have shown that these interactive cardiac effects would not be substantial in normal physiological ranges [53]. Due to these uncertainties however, the relationship between EDA and ostensible sympathetic HRV variables was further examined with the use of Cohen's kappa coefficient, which can determine the inter-rater agreement between two variables. As can be seen in Table 4, the complete lack of correlation between the reactivity of these two types of variables, when examined further, becomes fully apparent. A false negative indicates a case where electrodermal activity displayed the expected statistically significant increase in response to cognitive or orthostatic stress; while the activity of the ostensible sympathetic HRV marker decreased in response to either stressor. False negatives are reported as the percentage of the negative responses exhibited by that marker that were false. As highlighted by the number of false negatives in Table 4 (in response to both cognitive and orthostatic stress) the agreement between these two
types of markers is surprisingly low. This is further confirmed by low (and in some cases negative) kappa values, suggesting that no inter-rater agreement exists between electrodermal activity and the ostensible sympathetic HRV markers used in this study.

Table 4. Inter-rater agreement between reactivity of ostensible sympathetic HRV markers and EDA reactivity

<table>
<thead>
<tr>
<th></th>
<th>Cognitive stress</th>
<th>Orthostatic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>False (-)</td>
<td>Agreement (%)</td>
</tr>
<tr>
<td>ΔLF (ms²)</td>
<td>100% (23/23)</td>
<td>56.1</td>
</tr>
<tr>
<td>ΔLF (nu)</td>
<td>96% (23/24)</td>
<td>63.2</td>
</tr>
<tr>
<td>ΔVLF (ms²)</td>
<td>96% (23/24)</td>
<td>50.9</td>
</tr>
</tbody>
</table>

The above indications are in support of the findings of Reyes del Paso et al [16] who, in a relatively recent review on the influence of pharmacological and other manipulations of the autonomic nervous system, highlighted a number of pertinent issues why the LF band could not represent sympathetic nervous system activity.

The lack of correlation between VLF and EDA reactivity suggests that VLF does not represent sympathetic cardiac modulation as suggested by a number of recent papers [2,31–34]. This contradicts the paper by Shaffer et al [2] which suggests that the amplitude and frequency of the VLF rhythm is modulated by efferent sympathetic activity. Furthermore, although some authors have argued that VLF actually represents parasympathetic outflow to the heart [23,88] VLF did not correlate with HF or RMSSD in response to either stressor in this study (results not reported). This therefore suggests that VLF power does not represent either parasympathetic or sympathetic cardiac activity and might instead, as initially believed [1], be influenced by the renin-angiotensin system, and therefore be related to kidney functioning and thermoregulation, as previously suggested [1,88–90].

Origin of LF power
The parasympathetic nervous system has been shown to affect heart rhythms down to 0.05 Hz and parasympathetic activity can easily generate oscillations in the cardiac rhythms that cross
over into the LF band, especially during periods of low respiratory rates [2]. This therefore suggests that the HRV power spectrum, including its LF component, is in fact determined by the activity of the parasympathetic nervous system [16]. As previously discussed, some authors have thus postulated that variations within the LF range of the HRV spectrum reflect the vagal component of the baroreflex [16] and that manipulations that alter LF power do so through the modulation of cardiac autonomic outflow by baroreflex components [12]. Several newer studies, as reviewed by Goldstein et al [12], also support the concept that LF power, with or without respiratory adjustment, reflects baroreflex modulation (which is primarily vagal-mediated [2]), and not cardiac sympathetic tone [91]. In support of this, a statistically significant correlation between the reactivity of LF and the reactivity of universally used markers of parasympathetic function (i.e. HF and RMSSD) in response to orthostatic stress was found in the present study. This correlation was absent in response to the cognitive stressor, supporting the suggestion by a number of authors that LF represents baroreflex-cardiovagal function. It appears that LF is indeed able to index the modulation of cardiac vagal outflow by baroreflexes.

Conclusions
This study supports the conclusions by Reyes del Paso et al [16] and Rahman et al [91] that LF does not, in any way, represent cardiac sympathetic modulation and, furthermore, corroborates the finding that the LF component may reflect vagally-mediated baroreflex cardiac effects. Similarly, this study suggests that the VLF range of the HRV too does not appear to represent cardiac sympathetic modulation, as currently being interpreted by some in the literature. As highlighted by Reyes del Paso et al [16], HRV linear analysis currently appears to be restricted to the determination of vagal influences on HR, and interpretations of spectral components as extra-vagal have to be regarded as misleading. Therefore, in order to determine the activity or reactivity of the sympathetic nervous system in HRV research, a renowned sympathetic marker such as electrodermal activity needs to be included in the study. With regards to HRV parasympathetic markers, this study supports the suggestion that HRV frequency domain analyses, such as HF, should not be used as an index of vagal activity in study tasks where verbal responses are required as they may induce respiratory changes great enough to distort HF power.
Limitations

Since potential respiratory-linked changes in HF results of some of the participants were reported, it would have benefited the study if respiratory frequencies had been recorded. This would have allowed the comparison of respiratory frequency during each condition.

Author Contributions Statement

1. B Thomas – conception, design, acquisition, analysis and interpretation of data; drafting and revising of manuscript.

2. N Claassen – analysis and interpretation of data; revising of manuscript.

3. P Becker – analysis and interpretation of data; revising of manuscript.

4. M Viljoen – conception, design, analysis and interpretation of data; revising of manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Statement of Ethics

The study protocol was approved by the University of Johannesburg’s Faculty of Health Sciences Research Ethics Committee (approval number AEC01-31-2014), as well as the University of Pretoria’s Faculty of Health Sciences Research Ethics Committee (339/2014), and was conducted in accordance with the Declaration of Helsinki guidelines. Written informed consent was obtained from all participants.

References


2. Shaffer F, McCraty R, Zerr CL: A healthy heart is not a metronome: an integrative review of the heart’s anatomy and heart rate variability. Front Psychol 2014;5. DOI: 10.3389/fpsyg.2014.01040


5. Laborde S, Mosley E, Thayer JF: Heart Rate Variability and Cardiac Vagal Tone in Psychophysiological Research - Recommendations for Experiment Planning, Data Analysis, and Data Reporting. Front Psychol 2017;8:213.


12 Goldstein DS, Bentho O, Park M-Y, Sharabi Y: Low-frequency power of heart rate variability is not a measure of cardiac sympathetic tone but may be a measure of modulation of cardiac autonomic outflows by baroreflexes. Exp Physiol 2011;96:1255–1261.


20 Negrao BL: Autonomic correlates at rest and during evoked attention in children with attention-deficit/hyperactivity disorder and effects of sympathomimetic medication 2009 [cited 2015 Dec 3]; Available from: http://repository.up.ac.za/handle/2263/26102


22 Lutfi MF, Elhakeem RF: Effect of fasting blood glucose level on heart rate variability of healthy young adults. PLoS ONE 2016;11. DOI: 10.1371/journal.pone.0159820

23 Musa SM, Adam I, Lutfi MF: Heart rate variability and autonomic modulations in preeclampsia. PLoS ONE 2016;11. DOI: 10.1371/journal.pone.0152704


Posada-Quintero HF, Chon KH: Frequency-domain electrodhysical activity index of sympathetic functionLas Vegas, NV, USA, IEEE, 2016. DOI: 10.1109/BHI.2016.7455943


