Vagal contributions to fetal heart rate variability: an omics approach

Christophe L. Herry\textsuperscript{1,6}, Patrick Burns\textsuperscript{1,6}, André Desrochers\textsuperscript{1}, Gilles Fecteau\textsuperscript{1}, Lucien Daniel Durosier\textsuperscript{3}, Mingju Cao\textsuperscript{3}, Andrew J. E. Seely\textsuperscript{2} and Martin G. Frasch\textsuperscript{1,4,5,7}

\textsuperscript{1} Clinical Sciences, CHUV, University of Montréal, St-Hyacinthe, QC, Canada
\textsuperscript{2} Ottawa Hospital Research Institute, University of Ottawa, ON, Canada
\textsuperscript{3} Departments of Obstetrics and Gynaecology and Neurosciences, CHU Ste-Justine Research Centre, University of Montréal, QC, Canada
\textsuperscript{4} Centre de recherche en reproduction animale (CRRA), University of Montréal, St-Hyacinthe, QC, Canada
\textsuperscript{5} Department of Obstetrics and Gynecology, University of Washington, Seattle, WA, United States of America
\textsuperscript{6} PB and CH contributed equally to this manuscript.
\textsuperscript{7} Author to whom any correspondence should be addressed.
E-mail: mfrasch@uw.edu (M G Frasch)

Keywords: fetal monitoring, vagus nerve, fetal surgery, HRV

Abstract

Objective: Fetal heart rate variability (fHRV) is an important indicator of health and disease, yet its physiological origins, neural contributions, in particular, are not well understood. We aimed to develop novel experimental and data analytical approaches to identify fHRV measures reflecting the vagus nerve contributions to fHRV. Approach: In near-term ovine fetuses, a comprehensive set of 46 fHRV measures was computed from fetal pre-cordial electrocardiogram recorded during surgery and 72 h later without (n = 24) and with intra-surgical bilateral cervical vagotomy (n = 15). Main results: The fetal heart rate did not change due to vagotomy. We identify fHRV measures specific to the vagal modulation of fHRV: multiscale time irreversibility asymmetry index (AsymI), detrended fluctuation analysis (DFA) $\alpha_1$, Kullback–Leibler permutation entropy (KLPE) and scale-dependent Lyapunov exponent slope (SDLE $\alpha$). Significance: We provide a systematic delineation of vagal contributions to fHRV across signal-analytical domains which should be relevant for the emerging field of bioelectronic medicine and the deciphering of the ‘vagus code’. Our findings also have clinical significance for in utero monitoring of fetal health during surgery.

Key points
- Fetal surgery causes a complex pattern of changes in heart rate variability measures with an overall reduction of complexity or variability.
- At 72 h after surgery, many of the HRV measures recover and this recovery is delayed by an intrasurgical cervical bilateral vagotomy.
- We identify HRV pattern representing complete vagal withdrawal that can be understood as part of ‘HRV code’, rather than any single HRV measure.
- We identify HRV biomarkers of recovery from fetal surgery and discuss the effect of anticholinergic medication on this recovery.

Introduction

Variability is defined as patterns of fluctuation of a data series occurring over intervals in time. Various aspects of variability can be measured mathematically in several complementary domains, and hundreds of techniques of variability analysis have been proposed in the medical and broader literature (Electrophysiology, Task Force of the European Society of Cardiology and The North American Society of Pacing 1996, Bravi \textit{et al} 2011, Sassi \textit{et al} 2015). While some of these techniques capture properties of time series’ variability that are correlated,
most offer a unique aspect of variability by characterizing different mathematical properties of the signals. They include measures characterizing the statistical properties (e.g. standard deviation, root mean square of successive differences of R–R intervals of electrocardiogram (ECG), RMSSD), the informational complexity (e.g. entropy measures), the pattern of variations across time scales (i.e. time-invariant features such as fractal measures, power law exponents) or the energy contained in the signal (e.g. spectral measures). Researchers generally agree that a plurality of techniques offers the most complete evaluation (Goldberger 1996, Goldberger et al 2002).

An important underlying assumption about studying biological variability is that it has predictive properties about physiological systems from which it emerges. Hence, variability properties are a form of biological code, taking place on different scales in space and time. While the time component can be taken literally, the space component can represent different organs and regions of the body contributing to the properties of a variability pattern in question. The space component can also represent state space in terms of physical signal properties. The complexity of variability encompasses the regularity, degree of information and scale-invariant, self-similar fractal properties of physiological signals and offers a window into the temporal and spatial changes in structure and connectivity of complex dissipative systems. The degree of variability represents the amount of variation and relates to the physiological adaptability of the body, for instance, the ability to augment or decrease work as required by external demands (Carrillo et al 2016, Wang et al 2018).

Here we are concerned with a particular kind of biological variability characterized by complex, dynamic spatiotemporal patterns, the heart rate variability (HRV). HRV is an important indicator of health and disease and decreased variability is associated with age and illness and correlates with illness severity, indicating reduced adaptability and/or increased stress (Glass 2001, Seely and Macklem 2004, Macklem 2008, Macklem and Seely 2010, Leicht et al 2018). The physiologic interpretation of variability in time series and HRV, in particular, continues to be actively explored, impeded by the fact that HRV represents a complex, nonlinear, integration of activities of the sympathetic and parasympathetic nervous systems, influenced by body and breathing movements, baroreflex and circadian processes as well as various, quantitatively poorly understood homeokinetic brain–body communication processes, such as sensing and regulation of cytokine levels or metabolism (Thayer and Sternberg 2006, Jensen et al 2009, Tomokazu et al 2009, Durosier et al 2015, Liu et al 2016, Zanos et al 2018).

In HRV, we find features of spatial variability represented by contributions from various organs and systems such as heart, lung, brain, vasculature and endocrine system. We also find clear characteristics of state space variability in the above sense as well as rich temporal and time-invariant, fractal dynamics. Much like the established notion of neural code and the ongoing attempts to crack it (Borst and Theunissen 1999, Birmingham et al 2014, Bouton 2017), the notion of HRV code seems hence inevitable and logical to introduce and pursue.

While early HRV literature focused on the link between time-domain (e.g. RMSSD) and frequency domain (LF, HF) HRV measures and autonomic modulation (Electrophysiology, Task Force of the European Society of Cardiology and The North American Society of Pacing 1996, Montano et al 1998), it is clear that estimating autonomic activity with these simple linear metrics is limited and complicated by the previously mentioned nonlinear interconnection of complex sub-systems and processes (Hopf et al 1995, Skinner et al 2000, Frasch et al 2009, Billman 2011). Another approach promoted by Seely et al is to try and quantify the degree and complexity of variability as separate, yet complementary coupled concepts (Seely and Macklem 2012).

It is commonly held that HRV provides a window into vagal control: the effects of vagal modulation on some linear and nonlinear HRV properties have been reported (Tulppo et al 2001, 2005, Cerutti et al 2009, Frasch et al 2009) The assessment of sympathetic contributions has remained elusive, in part at least due to the complex overlap of vagal and sympathetic oscillations in the low frequency/long term time scales, albeit some studies indicated that complexity properties of HRV can reveal influences specific to sympathetic modulations (Frasch et al 2009).

While in adult physiology and medicine, ECG- or blood pressure-derived HRV represents one of many non-invasive monitoring modalities of health and disease, in fetal monitoring, HRV, derived from maternal abdominal ECG or from cardiac ultrasound, provides the only non-invasive window into fetal wellbeing or early detection of disease (Frasch 2018).

While attempts to assign some fHRV properties to vagal contributions have been made over the years (Karin et al 1993, Groome et al 1994, Ramaekers et al 2002, Thayer and Sternberg 2006, Frasch et al 2007, 2009, Fairchild et al 2011), it is not known how the complex communication via the vagus nerve influences fHRV’s complex properties, the HRV code.

We aimed to identify a comprehensive set of fHRV measures dependent on the intact vagal innervation. We modeled this condition using fetal sheep surgeries, a major homeokinetic disruptor, without or with a bilateral cervical vagotomy. We hypothesized that surgical intervention is associated with changes in measures of fHRV and vagotomy alters this association shedding further light onto the contributions of vagal activity fluctuations to the fHRV properties and maintenance of homeokinesis (Iberall and McCulloch 1968, Thayer and Sternberg 2006, Macklem and Seely 2010) We use this experimental approach to identify a subset of fHRV measures that
characterize vagal contributions, the vagus code (Kwan et al 2016). We present a methodological toolbox to do that borrowing from methodologies used in genomics.

**Methods**

Animal care for the *in vivo* approach followed the guidelines of the Canadian Council on Animal Care and the approval by the University of Montreal Council on Animal Care (protocol #10-Rech-1560).

**Anesthesia and surgical procedure**

We instrumented pregnant time-dated ewes at 126 d of gestation (dGA, ~0.86 gestation) with arterial, venous and amniotic catheters and ECG electrodes as described before (Burns et al 2015). For anaesthesia, the ewes were premedicated with acepromazine (Atravet 10 mg ml$^{-1}$) 2 mg intravenously; 30 min later, the animals were induced with a combination of diazepam (Diazepam 5 mg ml$^{-1}$) 20 mg, ketamine (Ketalar 100 mg ml$^{-1}$) 4–5 mg kg$^{-1}$ and Propofol (Propofol 10 mg ml$^{-1}$) 0.5 to 1 mg kg$^{-1}$. An endotracheal tube was inserted orally and ewes were ventilated in order to maintain a $P_{2}CO_{2}$ of 35 to 45 mmHg. Anaesthesia was maintained using isoflurane in oxygen such that the end-tidal isoflurane was 1.1 to 1.5%. The balanced poly-ionic solution was administered at 10 ml kg$^{-1}$ for the first hour of general anesthesia and then reduced to 5 ml kg$^{-1}$/h.

Ovine singleton fetuses of mixed breed were surgically instrumented with sterile technique under general anesthesia (both ewe and fetus). In the case of twin pregnancy, the larger fetus was chosen based on palpating and estimating the intertemporal diameter. The total duration of the procedure was carried out in about 2 h. Antibiotics were administered to the mother intravenously (Trimethoprim sulfadoxine 5 mg kg$^{-1}$) as well as to the fetus intravenously and into the amniotic cavity (ampicillin 250 mg). Amniotic fluid lost during surgery was replaced with warm saline. The catheters exteriorized through the maternal flank were secured to the back of the ewe in a plastic pouch. Buprenorphine 0.3 mg (0.3 mg ml$^{-1}$) was administered intravenously immediately following extubation. For the duration of the experiment, the ewe was returned to the metabolic cage, where she could stand, lie and eat ad libitum while we monitored the non-anesthetized fetus without sedating the mother. During post-operative recovery antibiotic administration was continued for 3 d. Arterial blood was sampled for evaluation of the maternal and fetal condition and catheters were flushed with heparinized saline to maintain patency.

During surgery (once the first fetal arterial brachial catheter was in place and before returning the fetus to the uterus) a 3 ml fetal arterial blood sample was taken for blood gas, lactate and base excess (BE) (ABL800Flex, Radiometer) and cytokine measurements. Precordial fetal ECG was placed as the first step of fetal instrumentation and recorded for the duration of surgery.

Bilateral cervical vagotomy was performed in selected sheep fetuses as follows. The head was extended and the neck was kept straight and stable while approaching and exposing the vagal nerves. We then performed a bilateral cervical vagotomy while recording fetal ECG. The skin was closed in a continuous pattern. The animals not subjected to vagotomy underwent a sham neck surgery with all steps except vagotomy (sham group).

**Experimental protocol**

Post-operatively, all animals were allowed 3 d to recover before starting the experiments. On these 3 d, at 9.00 am 3 ml arterial plasma sample was taken for blood gas analysis. For the purposes of this study, each experiment was completed at 9.00 am with a 1 h baseline measurement followed by the respective intervention as reported elsewhere (Burns et al 2015, Durosier et al 2015). After the +54 h (Day 3) sampling, the animals were sacrificed. The 72 h post-surgery, i.e. 1 h baseline, time point is reasonable to consider as a post-surgical recovery since the pharmacological effects of the anesthetic with respect to the cardiovascular system would have worn off by then. However, there may still be an effect caused by the anesthesia on the immune system which would be difficult to identify. We have previously shown that this surgical procedure does not result in systemic fetal inflammation (Burns et al 2015).

The animals reported in this study went on to be studied for an additional 72 h and were then killed with an intravenous injection of 20 ml sodium pentobarbital to the ewe as reported elsewhere (Burns et al 2015).

**FHRV analysis**

To derive fHHRV, fetal ECG recordings were studied over 5 min periods, starting 30 min prior to vagotomy and 15 min after vagotomy to ensure stable recordings. Thus, fHHRV was analyzed at three time points (see experimental diagram in figure 1): (1) before and (2) after vagotomy during surgery or at the equivalent point in time of surgery in sham animals followed (3) by a baseline measurement 72 h after surgery. The CIMVA (continuous individualized multiorgan variability analysis) software engine was used to develop comprehensive continuous fHHRV measures analysis (Herry et al 2013). Briefly, the analysis was as follows. First individual heartbeats were identified, using commonly used algorithms a time series of R-peak to R-peak intervals (RRI) was formed. A thorough automated assessment was performed of the quality of the underlying physiological
waveform signal and derived physiological events time series, based on the morphology of the ECG/respiratory waveforms, the level of noise/artifacts (including baseline drifts, spikes, and movement artifacts), the proportion of disconnected/saturated periods, and the presence of non-stationarity. Using the cleaned RRI time series, the signal complexity and degree of variability were assessed, through a moving window analysis, whereby a window of fixed duration (5 min) is shifted in time across the entire duration of the event time series. A comprehensive set of variability metrics are calculated within each window. The abbreviation list of all fHRV measures we calculated is provided in the table S1 (stacks.iop.org/PM/40/065004/mmedia). This analysis is performed iteratively and repetitively, measuring variability over time intervals. Only high-quality (as per the automated quality assessment) 5 min-windows were retained for the subsequent analysis.

**Statistical analysis**

Generalized estimating equations (GEE) modeling was used to assess the effects of treatment (sham, vagotomy) while accounting for repeated measurements on fetal blood gases, lactate and base excess (BE). We used a linear scale response model with time and treatment as predicting factors to assess their interactions using maximum likelihood estimate and Type III analysis with Wald Chi-square statistic. Table S1 presents the complete list of fHRV measures studied using CIMVA approach and their classification into signal-analytical domains and aspect of variability measured (degree or complexity of fHRV). As the CIMVA panel considered 46 HRV measures, each compared twice, a test-wise correction for multiple comparisons was made after performing two-sided Wilcoxon rank sum test at 5% significance level corrected with a false-discovery rate method of Benjamini–Hochberg–Yekutieli. To assess a median difference between the sham and vagotomy groups, we compared the three time points (surgery start, surgery end, and 72 h recovery) expressed as gain scores, i.e. the pairwise difference between the time points. SPSS Version 21 was used for these analyses (IBM SPSS Statistics, IBM Corporation, Armonk, NY). The results are presented as Mean ± SD, for P < 0.05.

**Results**

**Fetal arterial blood gas and acid-base status**

We found a significant effect of the main term time for all variables (observations prior and after surgery and 72 h recovery, P = 0.000, table 1). We found a significant effect of the main term vagotomy for pH, O2Sat, BE (P = 0.000, P = 0.010, P = 0.000, respectively) and no interaction between time and vagotomy (time * vagotomy, P = 0.210, P = 0.803, P = 0.054). There was no significant effect for pCO2, pO2 and lactate for vagotomy (P = 0.685, P = 0.672, P = 0.335); for pCO2 there was a significant interaction (P = 0.024). For pO2 and lactate the interaction term was not significant (P = 0.558 and P = 0.856). In summary, pH, O2Sat and BE showed similar recovery dynamics but were higher in vagotomy than sham animals. That is, vagotomy group animals were slightly less acidic than the sham animals.

---

Figure 1. Experimental approach. ECG was analysed from 15 min segments taken 15 min prior to and 15 min after vagotomy during the surgical instrumentation as well as 72 h after surgical recovery.
Vagotomy limits the fHRV recovery and identifies a subset of four fHRV measures that are affected by vagotomy.

At 72 h recovery compared to surgery onset, we observed group-specific fHRV changes. For sham animals, 25 out of 46 fHRV measures were changed by surgery with 20 measures showing increased complexity or variability and five measures showing a loss of complexity or variability. For vagotomy animals, we observed a change in five fHRV measures of which one showed increased complexity or variability and four showed a loss of complexity or variability.

When comparing to surgery end, at 72 h recovery we saw a similar pattern with 29 fHRV measures changed in sham animals of which 24 measures showed increased complexity or variability, while five showed a loss of complexity or variability. In vagotomy group animals, 12 fHRV measures changed of which eight measures showed increased complexity or variability, while four measures showed a loss of complexity of variability.

When comparing vagotomy versus sham group animals, most fHRV measures differed (33 out of 46) with six measures showing increased complexity or variability in vagotomy animals at 72 h recovery compared to sham animals and 27 measures showing loss of complexity or variability.

Next, to identify a core subset of fHRV measures reflecting HRV spontaneous activity, among the tested fHRV measures, we determined how many of the fHRV measures altered at 72 h recovery were specific to surgery with or without vagotomy. Figure 4 depicts the sets of fHRV measures specific or common to the effect of surgery (taken as 72 h recovery versus surgery start time) in both groups and the effect of vagotomy. To build the Venn diagrams we defined the physiological meaning of recovery in terms of fHRV changes as absence of significant difference at 72 h recovery versus surgery start time (figure 4(A)); we included those measures that rebounded significantly above the surgery start values, since variability and complexity increased, commonly associated with healthy states (figure 4(A)). We considered ‘the lack of recovery’ when the opposite condition was fulfilled, i.e. values significantly below the surgery start time point (figure 4(B)).

Figure 4(A) shows 42 and 41 measures that recovered in vagotomy and sham groups, respectively, with 37 measures being in common for both groups. Figure 4(B) shows two non-overlapping subsets of four measures that did not recover in either group.

While the LF/HF ratio increased, LF power and HF power were not significantly changed and therefore it is not clear whether the ratio indicates a true increase in variability with respect to our assumption and as a result LF/HF ratio was pooled with the four other measures that show a loss of complexity or variability.

Vagotomy limits the fHRV recovery and identifies a subset of four fHRV measures that are affected by vagotomy: AsymI, DFA α1, KLPE, and SDLE α.

Interestingly, the 20 fHRV measures that bounced back above values at the surgery start time point did not return to pre-surgery values during the subsequent 49 h of control conditions (previously reported (Durosier et al 2015)) possibly indicating that the impact of surgery on HRV is more long-lasting than previously thought.

Discussion

Little is known about the effects of fetal surgery on fHRV as a marker of fetal health and recovery from a major insult. We identified subsets of fHRV measures that can now be validated as fetal postsurgical recovery markers.
The relative difference between 72 h and start or end of surgery is overall smaller in vagotomy animals than in sham animals. Combined with findings from figure 4, we conclude that vagal denervation results in slower recovery from surgery. Our findings suggest that drugs influencing fetal vagal cholinergic signaling delay the HRV recovery and identify a set of four fHRV measures that reflect that effect: Asyml, DFA $\alpha_1$, KLPE, and SDLE $\alpha$.

While our study does not permit inference onto the categories of sympathetic or cardiovascular HRV contributions, we identified subsets of fHRV measures depending on intact vagus signaling. In the following we attempt two secondary analyses on existing fHRV data sets from related experiments (same gestational age): (a) contribution of the vagus nerve signaling to fHRV inflammatory signature and (b) the comparison to the recently identified intrinsic HRV (iHRV) signature.

**Comparison to systemic and organ-specific fHRV inflammatory signatures**

Via the vagus nerve, the brain senses systemic and organ-specific levels of inflammatory cytokines (Garzoni et al 2013, Zanos et al 2018). We showed that this process is reflected in specific subsets of HRV measures. In previous studies conducted in this cohort’s animals with intact vagus nerves (Durosier et al 2015, Herry et al 2016, Liu et al 2016), we identified a systemic LPS exposure response-specific fHRV signature as well gut- and

---

*Figure 2.* Fetal heart rate variability (fHRV) measures during and after surgery without (sham) and with vagotomy. Note no change in FHR in both groups, while a representative HRV measure Poincaré SD1 (equivalent to RMSSD) capturing short-term time scale HRV fluctuations shows a recovery at 72 h post-surgery in the sham, but not in vagotomy group. The comprehensive review of all changes in fHRV is presented in figure 5 (patterns).
Brain-specific HRV signatures (table S2). Here, we compared previously identified fHRV measures to those shown in this work to identify whether they change uniquely due to vagotomy or persist despite the vagotomy. We hypothesized that there would be minimal overlap between the vagotomy group in the present study and the inflammatory signatures because the latter represent a signaling process dependent on an intact vagus nerve (Garzoni et al. 2013).

To test this hypothesis, we examined the fHRV signature of vagotomy animals at 72 h of recovery to avoid contamination with acute post-surgical recovery effects. We found that the multiscale time irreversibility asymmetry index (AsymI) of fHRV, present in the systemic inflammatory index, did not recover in vagotomy group underscoring the connection of AsymI to vagus nerve signaling via the cholinergic anti-inflammatory pathway.

| Measure          | Surgery start | Surgery end | 72h |
|------------------|---------------|-------------|-----|
| ARerr            |               |             |     |
| SD1              |               |             |     |
| Teo              |               |             |     |
| SymDew_2         |               |             |     |
| SymDew_2         |               |             |     |
| gcount           |               |             |     |
| SymDse_2         |               |             |     |
| SymDp0_2         |               |             |     |
| SymDp1_2         |               |             |     |
| AsymI            |               |             |     |
| MSE              |               |             |     |
| KLPE             |               |             |     |
| QSE              |               |             |     |
| sgridAND         |               |             |     |
| CVI              |               |             |     |
| DFA α2           |               |             |     |
| HF Power         |               |             |     |
| SymDp2_2         |               |             |     |
| CSI              |               |             |     |
| dfmax            |               |             |     |
| pR               |               |             |     |
| sgridTAU         |               |             |     |
| sgridWGT         |               |             |     |
| vlnmax           |               |             |     |
| CD               |               |             |     |
| pD               |               |             |     |
| pl               |               |             |     |
| CVI              |               |             |     |
| DFA AUC          |               |             |     |
| SD2              |               |             |     |
| P5eo             |               |             |     |
| LF Power         |               |             |     |
| PLY-1            |               |             |     |
| PLS              |               |             |     |
| shannEn          |               |             |     |
| DFA α1           |               |             |     |
| SDLEx            |               |             |     |
| eScaleE          |               |             |     |
| histSI           |               |             |     |
| H. Comp          |               |             |     |
| MF_c2            |               |             |     |
| formF            |               |             |     |
| sedl             |               |             |     |
| sevl             |               |             |     |
| MF_c1            |               |             |     |
| LF/HF            |               |             |     |

Figure 3. Comparison of HRV at 72 h recovery to surgery start and end. Loss (blue) or increase (red) in variability or complexity for HRV measures that exhibited significant changes at 72 h recovery versus surgery start or surgery end time points. Grey cells represent non-significant changes. See table S1 for the abbreviations legend of the HRV measures.
(see figure 4(B)) (Garzoni et al 2013, Durosier et al 2015). DFA $\alpha_1$ did not recover in vagotomy animals as a measure shared with the organ-specific inflammatory index (for terminal ileum’s M2 (CD206$^+$) macrophages and Iba1-positive brain microglia—see table S2).

For the brain-specific inflammation signature, we found no further fHRV measures that reflected vagus nerve activity. Three out of five fHRV measures that did not recover in sham animals were also found in the brain inflammation signature: form factor (formF), multifractal spectrum cumulant of the first order (MultiFractal$c_1$) and similarity index of distributions (histSI). This supports our above-proposed notion of HRV spontaneous activity (figure 4(B)). The present findings suggest that is represented by formF, MultiFractal$c_1$, and histSI measures. Such activity can be perturbed either by brain inflammation or by the homeokinetic adaptations taking place after surgery.

**Comparison to iHRV signature**

In a recent study of fetal sheep heart ex vivo, i.e. devoid of any innervation, we identified a signature of iHRV activity in response to in vivo hypoxia (Frasch et al 2018b). The iHRV signature is comprised of two recurrence quantification metrics (dlmax, pl), a scale-dependent Lyapunov exponent metric (SDLE$\alpha$) and sgridAND, a grid transformation metric. Here we compared these iHRV measures to the fHRV measures reflecting lack of vagal innervation (figure 4(B)). We hypothesized that there would be an overlap in the fHRV measures in both groups, since they both represent iHRV, although other neural and non-neural extracardiac influences we could not control for in the present study likely played a role in the in vivo recorded fHRV with vagal denervation. Consequently, the fHRV measures that do not overlap may represent sympathetic or other, non-neural, influences on fHRV in the vagotomy group.

Figure 4(A) identifies a subset of fHRV measures recovering regardless of vagotomy that also belongs to the above iHRV signature: dlmax, pL, sgridAND. This finding strengthens the previously reported iHRV signature. For SDLE$\alpha$ which did not recover in vagotomized animals, we propose that in vivo formation of hypoxia memory in iHRV requires vagus-nerve-mediated imprinting on the iHRV signature. The behavior of these four fHRV measures should be studied longitudinally in vivo and ex vivo in future physiological studies.

**General discussion**

We propose to conceptualize the ensemble behavior of the multitude of the fHRV measures over time in terms of spontaneous and evoked activity reflected in HRV code, a time series of multi-dimensional dynamic patterns. We report here that the HRV code of the spontaneous activity overlaps considerably with the brain (but not systemic or gut’s) inflammation signature (see figure 4(B)). It may also be influenced by the regulatory activities of the cardiovascular and autonomic nervous systems and, more generally, suprabulbar brain’s efferent signaling via these systems. HRV’s response to a general insult of surgery can explain the overlap with the features of the brain inflammation signature of the HRV code.

The complexity of HRV pattern is highlighted by the finding that the five fHRV measures that did not recover in sham animals are distinct from those in the vagotomized animals (figure 4(B)). This supports the concept that we can identify specific HRV signatures of a general perturbation in the spontaneous activity as well as the HRV signature reflecting uniquely the withdrawal of vagal innervation.

We identified a set of fHRV measures characterizing recovery from surgery, a major injurious stimulus of general nature perturbing homeokinesis. The removal of vagus innervation altered the chronic recovery of the fHRV code represented by these measures. In other words, vagotomy perturbs such spontaneous activity further reflecting underlying HRV fluctuations which represent a proxy to the vagus code embedded in fHRV (Kwan et al 2016).
In sham animals, most fHRV measures recovered with complexity and variability increasing at 72 h when compared to pre or post-surgery time points. However, 30%–35% of fHRV measures showed a loss of complexity. If we are to account for collinearity in the measures, this subset of ‘dissenting’ measures constituted <10% (five measures in total). These ‘dissenting’ fHRV measures persisted for 1 week after surgery and might serve as ‘memory’ biomarkers of fetal surgery, although this would need to be tested further during longer observation periods. Another possible explanation might be the simplified interpretation of increase/loss of complexity/variability. For instance, the lower values of Hjorth’s Complexity and form factor (formF) generally indicate smoother (less complex) signals, but paradoxically can also relate to signals with a higher fractal dimension, suggesting that they characterize a different type of complexity compared to other metrics. Similarly, multifractal spectrum cumulants of the first and second order (Multifractal_c1, Multifractal_c2) are related to the maximum and width of the multifractal spectrum, respectively, and therefore characterize local regularity in the signal rather than complexity per se. The embedded scaling exponent (eScaleE) exhibited a very tight range in our data, so differentiating between groups with our relatively small sample size was difficult. Lastly, the similarity index (histSI) is a similarity measure between consecutive time segments and smaller values generally indicate segments are more dissimilar, although this does not always translate to higher intrinsic variability as that measure is affected by low-frequency components (Huang et al. 2008).

Vagotomized fetuses showed an overall pattern of chronic loss of complexity and overall degree of variability. We observed eight dissenting metrics that were significantly higher in vagotomy animals at 72 h compared with sham animals, most of them consistent with the previously discussed dissenting metrics with the exception of sedl and sevl (Shannon entropy of diagonal and vertical lines, respectively, from the recurrence plots) and LF/HF ratio. The increase in the Shannon entropy of diagonal and vertical lines could be due to noise effects, while the LF/HF ratio increase is most likely due to a drop in HF power.

For vagotomy animals, we observed some recovery of complexity or variability, but it was reflected by a different set of fHRV measures than for the sham animals. Comparing both groups at 72 h, our data shows that vagotomy hindered the recovery of complexity and variability after surgery. That is in line with the notion that vagus nerve is homeokinetic in nature, hence vagus’ withdrawal would be expected to interfere with the surgical recovery (Thayer and Sternberg 2006, Frasch et al. 2009). Here we identified a set of fHRV measures that reflect this effect, most of which are not yet conventionally used as biomarkers of vagal modulation of HRV.

When comparing with previous work on systemic and organ-specific fHRV inflammatory signatures, our findings indicate that AsymI might be uniquely tied to the vagus nerve signaling via the cholinergic anti-inflammatory pathway. This result is supported by the studies in septic neonates where AsymI is part of the HRV-derived inflammatory index to predict early onset of neonatal sepsis (Lake et al. 2014). To our knowledge, the present study is a first mechanistic demonstration of the connection between AsymI and vagus nerve signaling linking it to the cholinergic anti-inflammatory pathway (Andersson and Tracey 2012). Furthermore, both AsymI and DFA α1 HRV measures appear to be components of the vagus code dynamics in response to inflammation and can serve as biomarkers of inflammation and organ-specific vagus code signatures. Conversely, we observed the presence of HRV measures in the organ-specific inflammation signatures that do not map uniquely onto the contribution of the vagus nerve activity. Such behavior is to be expected and yields the more general HRV code, as the sensing and control of inflammation represents a complex homeokinetic process with nonlinear contributions from multiple physiological systems. We suggest that HRV code in general and vagus code in particular ultimately represent an emergent property reflecting the underlying communication via the vagus nerve and other complex oscillatory influences. As reviewed elsewhere (Kwan et al. 2016) and demonstrated in several recent studies (Steinberg et al. 2016, Datta-Chaudhuri et al. 2018), the organ specific vagus code can be captured directly via vagus nerve electroneurogram (VENG). The precise delineation of the relationships between VENG properties and HRV code properties is the subject of future studies.

In utero hypoxia likely reduces vagal tone in favor of sympathetic activity (Morrison 2008). If this imbalance is imprinted with a ‘1:1’ transfer function onto iHRV, we would expect a similar pattern with an increased percentage of Laminarity (pl) and maximum diagonal line (dlmax) in the Recurrence plots in ex vivo hypoxic hearts, but the opposite was found consistently. This suggests that the drop in pl and dlmax may reflect ‘true’ iHRV dynamics or the ex vivo memory of hypoxia is not retained in a linear fashion.

The present study has several limitations. First, the time points for assessment of the acute fHRV changes during surgery as a function of vagotomy were at 30 min relatively close to each other. However, we deliberately targeted stable pre- and post-vagotomy segments of the surgery which both shared the same anesthetic regime and surgical procedures, including a sham vagotomy in the sham experimental group. Second, we conducted a number of comparisons for fHRV measures in this study and adjusted for the multiple comparisons within each fHRV measure for each time point. As recommended by Rothman (1990), we did not adjust for the overall number of comparisons across all possible tests.

Second, we assumed that our set of HRV measures represented an adequate characterization of the variability of the complex cardiovascular system and attempted to reveal specific HRV signatures triggered by physiological
perturbations, inflammation, and iHRV/hypoxia. However, it is possible that these HRV signatures we identified underestimate the true dimensionality of the process, even if they correlate or match well with some of its dynamic features. Future studies should attempt to approach this problem directly, rather than inversely, e.g., by recording fetal VENG and sympathetic nerve activity and studying its properties in conjunction with changes in HRV code. We are preparing reports on the respective methodological approach and first physiological findings (Frank et al 2018, Frasch et al 2018a).

We consider it beyond the scope of the present study to elucidate the reasons for specific changes in each fHRV measure and leave it to future work. We hope that our current work raises awareness of paradoxical responses in fHRV measures. This should be considered during interpretation of the effects of reduced vagal modulation in other experiments. Our present work highlights the difficulty in summarizing fHRV changes as being more/less complex or suppressed/increased variability (Frasch 2018). The various HRV measures characterize different, sometimes conflicting, aspects of the signal and the dynamics of the underlying system. It is for this reason that we introduced the terms ‘HRV code’ and ‘vagus code’. The search for physiological meaning is ongoing. This highlights the continued need for collaborative transdisciplinary efforts to refine further the multi-dimensional/multidomain HRV concept toward the formulation of a robust mathematically-founded biological HRV code. We hope the present study contributes to this development.

Acknowledgments

Supported by CIHR, FRQS, Molly Towell Perinatal Research Foundation.

Conflict of interest statement

AJE Seely founded Therapeutic Monitoring Systems in order to commercialize patented continuous individualized multiorgan variability analysis (CIMVA) technology, with the objective of delivering variability-directed clinical decision support to improve quality and efficiency of care. CI. Herry is a patent holder related to waveform quality assessment necessary for variability analysis. MG Frasch holds a PCT on fetal ECG monitoring. All other authors declare that they have no conflict of interest.

References

Andersson U and Tracey K J 2012 Reflex principles of immunological homeostasis Annu. Rev. Immunol. 30 313–35
Billman G E 2011 Heart rate variability—a historical perspective Frontiers Physiol. 2 86
Birmingham K, Gradinaru V, Anikeeva P, Grill W M, Pikov V, McLaughlin B, Pasricha P, Weber D, Ludwig K and Famm K 2014 Bioelectronic medicines: a research roadmap Nat. Rev. Drug Discov. 13 399–400
Borst A and Theunissen F E 1999 Information theory and neural coding Nat. Neurosci. 2 947–57
Bouton C 2017 Cracking the neural code, treating paralysis and the future of bioelectronic medicine J. Intern. Med. 282 37–45
Bravi A, Longtin A and Seely A J E 2011 Review and classification of variability analysis techniques with clinical applications Biomed. Eng. 10 90
Burns P, Liu H L, Kuthiala S, Fecteau G, Desrochers A, Durosier L D, Cao M and Frasch M G 2015 Instrumentation of near-term fetal sheep for multivariate chronic non-anesthetized recordings J. Vis. Exp. 104 e52581
Carrillo A E et al 2016 Heart rate variability during high heat stress: a comparison between young and older adults with and without Type 2 diabetes Am. J. Physiol. 311 R669–75
Cerutti S, Hoyer D and Voss A 2009 Multiscale, multiorgan and multivariate complexity analyses of cardiovascular regulation Phil. Trans. A 367 1337–58
Datta-Chaudhuri T, Tsavva T, Addorissio M, Bouton C, Tracey K J and Chavan SS 2018 Selective electrical stimulation of vagus nerve induces specific cytokine response J. Physiol. 200 43.11
Durosier L D, Herry C L, Cortes M, Cao M, Burns P, Desrochers A, Fecteau G, Seely A J and Frasch M G 2015 Does heart rate variability reflect the systemic inflammatory response in a fetal sheep model of lipopolysaccharide-induced sepsis? Physiol. Meas. 36 2089–102
Electrophysiology, Task Force of the European Society of Cardiology and The North American Society of Pacing 1996 Heart rate variability: standards of measurement, physiological interpretation, and clinical use strategies to reduce sudden death among children with Wolff–Parkinson–White syndrome Circulation 93 1043–65
Fairchild K D, Srinivasan V, Meerman J R, Gaykema R P and Goehler L E 2011 Pathogen-induced heart rate changes associated with cholinergic nervous system activation Am. J. Physiol. 300 R330–9
Frank Y, Last M and Frasch M G 2018 Detection and treatment of fetal and newborn infections via heart rate monitoring and vagus nerve stimulation Reproductive Science (Thousand Oaks, CA: SAGE Publications Inc.) pp 213A–4A
Frasch M G 2018 Saving the brain one heartbeat at a time J. Physiol. 596 5503–4
Frasch M G, Burns P, Benito J, Cortes M, Cao M, Fecteau G and Desrochers A 2018a Sculpting the sculptors: methods for studying the fetal cholinergic signaling on systems and cellular scales Methods Mol. Biol. 1781 341–52
Frasch M G, Bird C, Niu Y and Giussani D A 2018b First evidence that intrinsic fetal heart rate variability exists and is affected by chronic hypoxia (Available at: https://doi.org/10.1101/242107)
Frasch M G, Mueller T, Hoyer D, Weiss C, Schubert H and Schwab M 2009 Nonlinear properties of vagal and sympathetic modulations of heart rate variability in ovine fetus near term Am. J. Physiol. 296 R702–7
Frasch M G, Mueller T, Wicher C, Weiss C, Loehle M, Schwab K, Schubert H, Nathanielsz P W, Witte O W and Schwab M 2007 Fetal body weight and the development of the control of the cardiovascular system in fetal sheep J. Physiol. 579 893–907
Garzoni I, Faure C and Frasch M G 2013 Fetal cholinergic anti-inflammatory pathway and necrotizing enterocolitis: the brain-gut connection begins in utero Frontiers Integr. Neurosci. 7 57
Glass I. 2001 Synchronization and rhythmic processes in physiology Nature 410 277–84
Goldberger A L 1996 Non-linear dynamics for clinicians: chaos theory, fractals, and complexity at the bedside Lancet 347 1312–4
Goldberger A L, Peng C-K and Lipsitz L A 2002 What is physiologic complexity and how does it change with aging and disease? Neurobiol. Aging 23 23–6
Groeume L J, Mooney D M, Bentz L S and Wilson J D 1994 Vagal tone during quiet sleep in normal human term fetuses Dev. Psychobiol.
Goldberger A L 1996 Non-linear dynamics for clinicians: chaos theory, fractals, and complexity at the bedside Handbook of Systems and Complexity in Health ed J P Sturmburg and C Martin (Berlin: Springer) pp 467–81
Hopf H R, Skyschally A, Heusch G and Peters J 1995 Low-frequency spectral power of heart rate variability is not a specific marker of cardiac sympathetic modulation Anesthesiology 82 609–19
Huang H H, Lee Y H, Chan H L, Wang Y P, Huang C H and Fan S Z 2008 Using a short-term parameter of heart rate variability to distinguish awake from isoflurane anesthetic states Med. Biol. Eng. Comput. 46 977–84
Iberall A S and McCulloch W S 1968 Homeokinetics—the organizing principle of complex living systems IFAC Proc. Vol. 2 39–50
Jensen E C, Bennet L, Guild S J, Booth L C, Stewart J and Gunn A J 2009 The role of the neural sympathetic and parasympathetic systems in diurnal and sleep state-related cardiovascular rhythms in the late-gestation ovine fetus Am. J. Physiol. 297 R998–1008
Karin J, Hirsch M and Akedrud S 1993 An estimate of fetal autonomic state by spectral analysis of fetal heart rate fluctuations Pediatr. Res. 34 134–8
Kwan H, Garzoni L, Liu H L, Cao M, Desrochers A, Fectue G, Burns P and Frasch M G 2016 VNS in inflammation: systematic review of animal models and clinical studies Bioelectrochem. Med. 3 1–6
Lake D E, Fairchild K D and Moorman J R 2014 Complex signals bioinformatics: evaluation of heart rate characteristics monitoring as a novel risk marker for neonatal sepsis J. Clin. Monit. Comput. 28 329–39
Leicht A S, Flouris A D, Kaltsatou A, Seely A J, Herry C L, Wright Beatty H E and Kenny G P 2018 Age alters cardiac autonomic modulations during and following exercise-induced heat stress in females Temperature 5 184–96
Liu H L et al 2016 Can monitoring fetal intestinal inflammation using heart rate variability analysis signal incipient necrotizing enterocolitis of the neonate? Pediatr. Care. Med. 17 e165–76
Macklem P T 2008 Emergent phenomena and the secrets of life J. Appl. Physiol. 104 1844–6
Macklem P T and Seely A 2010 Towards a definition of life Perspect. Biol. Med. 53 330–40
Montano N, Cogliati C, Porta A, Pagani M, Malliani A, Narkiewicz K, Abboud F M, Birkett C and Somers V K 1998 Central vagotonic effects of atropine modulate spectral oscillations of sympathetic nerve activity Circulation 98 1394–9
Morrison J L 2008 Sheep models of intrauterine growth restriction: fetal adaptations and consequences Clin. Exp. Pharmacol. Physiol. 35 730–43
Ramakers D, Beckers F, Demeulemeester H and Aubert A E 2002 Cardiovascular autonomic function in conscious rats: a novel approach to facilitate stationary conditions Ann. Noninvasive Electrocardiol. 7 307–18
Rothman K J 1990 No adjustments are needed for multiple comparisons Epidemiology 1 43–6
Sassi R, Cerutti S, Lombardi F, Malik M, Huiuki H V, Peng C-K, Schmidt G and Yamamoto Y 2015 Advances in heart rate variability signal analysis: joint position statement by the e-Cardiology ESC Working Group and the European Heart Rhythm Association co-endorsed by the Asia Pacific Heart Rhythm Society Europace 17 1341–53
Seely A J and Macklem P 2012 Fractal variability: an emergent property of complex dissipative systems Chaos 22 013108
Seely A J and Macklem P T 2004 Complex systems and the technology of variability analysis Crit. Care 8 R367–84
Skinner J E, Nester B A and Dalsey W C 2000 Nonlinear dynamics of heart rate variability during experimental hemorrhage in ketamine- anesthetized rats Ann. J. Physiol. 279 H1669–75
Sorani M D, Hemphill J C 3rd, Morabito D, Rosenthal G and Manley G T 2007 New approaches to physiological informatics in neurocritical care Neurocrit. Care. 7 45–52
Steinberg B E et al 2016 Cytokine-specific neurograms in the sensory Vagus nerve Bioelectrom. Med. 37 7–17
Thayer J F and Sternberg E 2006 Beyond heart rate variability: vagal regulation of allostatic systems Ann. New York Acad. Sci. 1088 361–72
Tomokazu T, Akira U, Eiji N, Mai H, Mariko H, Takashi K, Hisayuki U and Kunio T 2009 Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis Gastroenterology 137 262–73
Tulppo M P, Kiviniemi A M, Hautala A J, Kallio M, Seppänen M, Makikallio T H and Hukkuri H V 2005 Physiological background of the loss of fractal heart rate dynamics Circulation 112 314–9
Tulppo M P, Makikallio T H, Seppänen T, Shoemaker K, Tungui E, Hughson R L and Hukkuri H V 2001 Effects of pharmacological adrenergic and vagal modulation on fractal heart rate dynamics Clin. Physiol. 21 515–23
Wang G, Jia S, Li H, Wang Z and Zhang W 2018 Exploring the relationship between blood flux signals and HRV following different thermal stimulations using complexity analysis Sci. Rep. 8 89882
Zanos T, Silverman H, Levy T, Tsava T, Battinelli E, Lorraine P, Ashe J, Chavan S S, Bouton C and Tracey K J 2018 Identification of cytokine-specific sensory neural signals in murine vagus nerve activity recordings J. Immunol. 200 43.12