HEART RATE VARIABILITY AND OTHER AUTONOMIC MARKERS IN CHILDREN AND ADOLESCENTS

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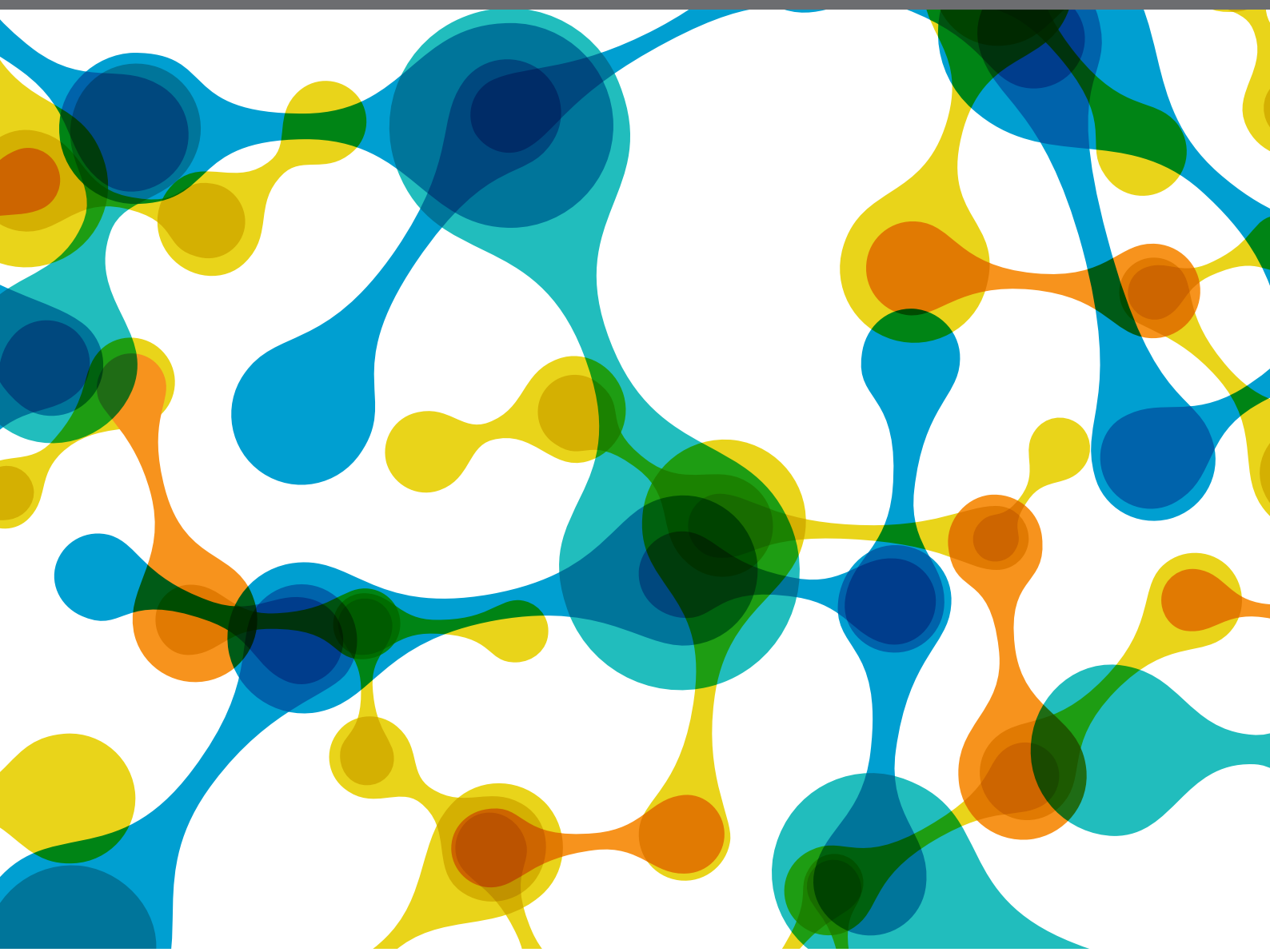

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HEART RATE VARIABILITY AND OTHER AUTONOMIC MARKERS IN CHILDREN AND ADOLESCENTS

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EDITORIAL
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Editorial: Heart Rate Variability and Other Autonomic Markers in Children and Adolescents

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Editorial on the Research Topic

Heart Rate Variability and Other Autonomic Markers in Children and Adolescents

Despite thousands of articles addressing heart rate variability (HRV) in healthy subjects and patients with various clinical conditions published during the last decades (Billman, 2011), our understanding of the development of the cardiac autonomic nervous system is still very limited. During maturation, from infancy through adolescence to adulthood, changes in cardiac autonomic neural regulation elicit corresponding changes in cardiac rhythm, changing both heart rate (HR) and its beat-to-beat variability. These fluctuations in heart rate display marked regularity, corresponding with changes in respiration (i.e. HR increases during inspiration and decreases with expiration), and are thought to reflect changes in a cardiac autonomic regulation (Billman, 2011). For example, it is widely accepted that the high frequency component of the R-R interval variability is dominated by changes in cardiac parasympathetic efferent nerve activity while the relationship, if any, between cardiac autonomic regulation and the low frequency component of this variability is much more complex and the subject of considerable debate (Houle and Billman, 1999; Billman, 2011, 2013a). Despite this controversy, various mathematical approaches have been developed, both to investigate cardiac autonomic regulation in healthy individuals and to identify changes that might be associated with an increased risk for adverse cardiac events (Billman, 2011). However, the majority of these studies have focused on changes in adult populations. Furthermore, it is not widely appreciated that, as a consequence of the non-linear inverse relationship between HR and R-R interval, similar changes in HR can provoke profoundly different values for HRV (i.e. the R-R interval variability) depending on the prevailing average HR: bigger values for HRV at a lower prevailing HR than at a higher HR (Sacha and Pluta, 2005; Billman, 2013b; Sacha, 2014a,b). Consequently, any changes in HR usually entail simultaneous changes in HRV.

The most common developmental phenomenon is a reduction of HR with a child's age, which in turn, may influence HRV. The extent to which alterations of HRV during growth and development result from this HR change remains to be determined. The normalization of HRV for the prevailing HR liberates HRV from the influence of HR and thereby allows for the objective assessment of the cardiac autonomic influences on this variability, separating mathematical from physiological changes in HRV (Sacha and Pluta, 2005; Billman, 2013b; Sacha, 2014a,b). Moreover, other autonomic markers such as deceleration capacity and indices of baroreceptor reflex sensitivity are

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also sensitive to changes in prevailing HR and could be effected by developmental changes in HR. It is therefore critical to assess both tonic and reflex autonomic markers independently of average HR in order to evaluate changes in cardiac autonomic regulation during maturation from the infant to the young adult.

As previously noted, there are both linear and non-linear components to HRV, yet the effects of development on non-linear heart rate dynamics remain to be determined. Although a number of non-linear autonomic markers have been proposed and tested in adult populations (Billman, 2011), little is known about either the effects of maturation on these markers or the effects of changes in prevailing HR during growth and development. It is also unclear whether cardiac autonomic function becomes more linear or non-linear with age in healthy children. Finally, the relationship between changes in indices of HRV during the maturation process and abnormalities/diseases in pediatric populations also remains to be determined. In other words, can changes in HR and its variability be used as part of the differential diagnosis process to identify specific pathologies and those individuals at the greatest risk for complications from disease, as has been proposed for adult populations (Billman, 2011)? It is the purpose of this monograph to provide a comprehensive assessment of developmental changes in both HR and HRV in infants, young children, and adolescents. Particular emphasis is placed on the contribution of HR to alterations in HRV during maturation in healthy populations. In addition, HRV indices are evaluated as potential markers for the identification of individuals at risk for pediatric diseases. The book is divided into the three main section: HRV in infants and young children (<3 years of age), young children (3–12 years of age) and adolescents to young adults (13–21 years of age). A brief summary of the chapters contained in each section follows.

The first section examines HRV in infants and young children. In chapter 2, Oliveira et al. evaluate changes in HRV during the first 24 h after birth, reporting that HRV increases during the first 6 h and then declines toward new steady state values. They further find a strong correlation between reduced HRV and clinical risk factors, concluding that HRV is a good indicator of overall well-being and can be used as a marker of birth-related stress. Chapter 3 compares the impact of paternal or maternal stroking touch on 4–16 week old infants, reporting that touch by either parent increased respiratory sinus arrhythmia amplitude (Van Puyvelde et al.). Chapter 4 closes this section, with an investigation of HRV for risk assessment in toddlers (18–36 months old) with and without developmental difficulties (i.e., social dysfunction). Billeci et al. report that the HRV response to a joint attention task (defined as the ability to coordinate visual attention with another person and then shift the gaze toward an object or event) was attenuated in toddlers exhibiting signs of social dysfunction (autism spectrum disorders) compared to typically developed children.

The second section, investigates HRV in young and pre-adolescent children. Chapter 5, Gąsior et al. examine a number of HRV indices in children ages 6–13 years (non-athletes) in order to establish the normal range of these values after correction for prevailing HR. Their study provides important normative values for these various HRV indices, finding that they vary independently of either gender or changes in HR. This chapter also provide a link to supplementary material that contains mathematical tools that can be used to correct HRV for HR. In a similar manner (chapter 6, Bobkowski et al.), investigate the association between age and gender on non-linear indices of HRV in young children and adolescents (ages 3–18 years). They report that these non-linear indices were not affected by gender but increased with age. Chapter 7–10 then evaluate the association between body composition and physical activity on HRV in children. For example, Herzig et al. (chapter 7), assess the association between physical activity and/or body mass on HRV after correction for age-related changes in HR in children aged 2–6 years. They report that HR and skin fold thickness both decrease with increased levels of habitual physical activity. Importantly, indices of HRV increased with age and activity only after adjustment for age-associated reductions in prevailing HR. In chapters 8 and 9, Plaza-Florido et al. further characterize the effects of HR on HRV in overweight and clinically obese children. They report that HRV declines with increasing weight. However, this weight-associated decline in HRV is eliminated by correction for prevailing HR (chapter 8, Plaza-Florido et al.). They (chapter 9, Plaza-Florido et al.) further demonstrate that physical activity reduces HR and increases HRV but, once again, the increase in HRV is eliminated by adjustment for HR. These results further emphasize that HRV indices must be adjusted for prevailing HR in order to reveal true differences or changes in cardiac autonomic regulation. The relationship between the energy-related biomarkers, leptin and adiponectin, and HRV in boys and girls is examined in chapter 10. Specifically, leptin was a negative predictor of "parasympathetic" regulation in boys and was associated with higher values of low frequency power in girls (Van De Wielle and Michels). In contrast, adiponectin was a negative predictor of HRV in girls but not in boys after adjustment for prevailing HR (Van De Wielle and Michels). Chapter 11 investigates the relationship between non-linear indices of HRV and psychological disorders in children aged 9–13 years. Fiskum et al. report that non-linear indices of HRV were inversely related to the severity of the psychopathology: higher values of HRV were associated with lower incidence of disorder. Similarly, children (mean age 8 years old) with developmental coordination disorders exhibit lower HRV in the supine position and a blunted response to an orthostatic challenge than did children without coordination disorders (chapter 12, Cavalcante -Neto et al.).

The final section examines HRV variability in adolescents and young adults. The results of a meta-analysis that included about 5,000 children aged 12–17 years are presented in chapter 13. In contrast to studies that include younger children, girls have higher prevailing HRs and lower HRV than boys (Koenig et al.). Two different approaches to reduce the effects of HR and HRV, using computer stimulation studies on data obtained from males aged 19–38 years, are compared in chapter 14. Both regression analysis formulae and interpolating an R-R interval time series effectively attenuated the effects of HR on HRV (Bolea et al.). This section closes with an examination of the effects of physical activity (chapter 15) on HR and HRV as well as an investigation of the relationship between HRV and internalizing disorders

(depression and anxiety) (chapter 16) or sleep stability (chapter 17). In a cross-sectional study of young athletes aged 10–19 years (Subramanian et al.), exercise is associated with lower baseline HR and increased indices of cardiac parasympathetic regulation, just like in younger children. Conversely, anxiety and depression were more common in adolescent girls than in similarly aged boys, and these psychological disorders were accompanied by lower HRV and cardiac complexity (Fiol-Veny et al.). The authors suggest that these indices may help identify individuals particularly sensitive to psychological stressors. Finally, Cysarz et al. investigate the association between aging related changes in sleep patterns (duration and stability) and HRV as measured by cardiopulmonary coupling analysis (CPC, the degree of coherent coupling between HRV and variations in the amplitude of the R wave produced by changes in tidal volume that occur during respiration) in children and young adults (ages 4–22 years). They find that both sleep stability and CPC decline with age.

The authors hope that this brief monograph will serve as a guide to how to use linear and non-linear indices of HRV appropriately (i.e., after adjustment for age-related changes in HR). Both research investigators and clinicians will then be able to use HRV indices not only to evaluate developmental changes in cardiac regulation but also to aid in the identification and treatment of individuals at risk for pediatric diseases.

AUTHOR CONTRIBUTIONS

The editorial was written by GB and proof read and approved by the co-authors for submission.

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ORIGINAL RESEARCH
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Early Postnatal Heart Rate Variability in Healthy Newborn Infants

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Background: Despite the increasing interest in fetal and neonatal heart rate variability (HRV) analysis and its potential use as a tool for early disease stratification, no studies have previously described the normal trends of HRV in healthy babies during the first hours of postnatal life.

Methods: We prospectively recruited 150 healthy babies from the postnatal ward and continuously recorded their electrocardiogram during the first 24 h after birth. Babies were included if born in good condition and stayed with their mother. Babies requiring any medication or treatment were excluded. Five-minute segments of the electrocardiogram (non-overlapping time-windows) with more than 90% consecutive good quality beats were included in the calculation of hourly medians and interquartile ranges to describe HRV trends over the first 24 h. We used multilevel mixed effects regression with auto-regressive covariance structure for all repeated measures analysis and *t*-tests to compare group differences. Non-normally distributed variables were log-transformed.

Results: Nine out of 16 HRV metrics (including heart rate) changed significantly over the 24 h [Heart rate $p < 0.01$; Standard deviation of the NN intervals $p = 0.01$; Standard deviation of the Poincaré plot lengthwise $p < 0.01$; Cardiac sympathetic index (CSI) $p < 0.01$; Normalized high frequency power $p = 0.03$; Normalized low frequency power $p < 0.01$; Total power $p < 0.01$; HRV index $p = 0.01$; Parseval index $p = 0.03$], adjusted for relevant clinical variables. We observed an increase in several HRV metrics during the first 6 h followed by a gradual normalization by approximately 12 h of age. Between 6 and 12 h of age, only heart rate and the normalized low frequency power changed significantly, while between 12 and 18 h no metric, other than heart rate, changed significantly. Analysis with multilevel mixed effects regression analysis (multivariable) revealed that gestational age, reduced fetal movements, cardiotocography and maternal chronic or pregnancy induced illness were significant predictors of several HRV metrics.

Conclusion: Heart rate variability changes significantly during the first day of life, particularly during the first 6 h. The significant correlations between HRV and clinical risk variables support the hypothesis that HRV is a good indicator of overall wellbeing of a baby and is sensitive to detect birth-related stress and monitor its resolution over time.

Keywords: heart rate variability, healthy, term, infant, newborn

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INTRODUCTION

Heart rate variability (HRV) analysis provides insights into autonomic regulation and the interactions between sympathetic and parasympathetic nervous systems. HRV describes the variations in heart rate over time that occur naturally in healthy states. Those variations reflect the organism's ability to continuously adjust to internal and external events, in order to maintain homeostasis. Interestingly, Chrousos and Gold (1992, p. 1245) defined stress as a "*state of threatened homeostasis*". Therefore, over the years a decrease of HRV has been presumed to reflect an elevation in stress and HRV analysis has been increasingly recognized as one of the methodologies for measuring stress.

One of the simplest HRV measurements (SDNN) quantifies the standard deviation of the duration of normal RR intervals, i.e., how the interval between normal (sinus) R-peaks of consecutive QRS's on the electrocardiogram (ECG) varies over time. Nonetheless, numerous mathematical metrics and approaches to HRV analysis have been developed over the years to extract more and more accurate information from HRV (Shaffer and Ginsberg, 2017).

Time domain HRV analysis is focused on the variation of the NN intervals (i.e., normal RR intervals) over time. In addition to the SDNN, HRV studies frequently examine the root mean square of successive differences (RMSSD) or the percentage of intervals that differ from the previous by more than 50 ms (pNN50) or 20 ms (pNN20). For all these HRV metrics in the time-domain, higher values reflect higher variability, which is more prevalent in healthy states.

Non-linear or geometric HRV analysis can be performed by plotting the NN-intervals on a Poincaré plot, where each NN interval is plotted in relation to the previous NN-interval (Golińska, 2013) and the standard deviation of the main cluster of data-points is measured crosswise (SD1) or lengthwise (SD2). Metrics such as the Cardiac Sympathetic Index (CSI) and Cardiac Vagal Index (CVI) have been developed to reflect the interactions between SD1 and SD2 (Toichi et al., 1997). CSI behaves in opposition to CVI, therefore, unlike most other HRV metrics, higher CSI is associated with lower variability, i.e., higher stress. Other geometric metrics include the Triangular Index (TINN) and the HRV Index. Like the SDNN, these two parameters indicate a measure of overall variability during the recording period. The TINN measures the normalized width of the base of histogram of the NN intervals (in relation to the highest value of the NN histogram) and the HRV Index is a ratio between the number of all NN intervals and the number of NN intervals at the highest point of the NN histogram (normalized to a sampling rate of 128 values per second).

In frequency domain analysis, different bands of the ECG power spectrum are analyzed as well as their interactions (between bands and in relation to the total power). In adults, previous studies have defined four frequency bands of interest: Ultra-low frequency (ULF), Very-low frequency (VLF), Low-frequency (LF), and High-frequency (HF), each of which is deemed to have different physiological origins. ULF has been associated with circadian oscillations of core body temperature and renin-angiotensin regulation; VLF has been associated with longer-term regulation of thermoregulation and hormonal mechanisms; LF has been associated with a mix of sympathetic and vagal activity and baroreceptor activity and HF has been associated with vagal activity (Pomeranz et al., 1985). Nonetheless, the definition and meaning of ULF and VLF in babies is under-documented and, therefore, is not included in this report. Although absolute quantifications of power in HF and LF bands can increase/decrease, under normal cardiac conductivity we expect LFn and HFn (which are LF and HF normalized to total power) to behave in opposite directions in most cases. Therefore, whereas HFn (representing parasympathetic activity) is expected to be higher when physiological stress is low, LFn is expected to be higher when stress is high. These associations are, nonetheless, controversial (von Rosenberg et al., 2017; Adjei et al., 2019) and the interpretation of LF and HF findings in real-life scenarios requires caution.

Heart rate variability analysis has been extensively accepted as a method to measure autonomic impairment and increasingly examined with regards to its value in disease stratification (Ahmad et al., 2009; Lees et al., 2018; Oliveira et al., 2018). Although previous studies have described normative HRV reference values for newborns in the first few days (Mehta et al., 2002; Longin et al., 2005; Doyle et al., 2009; Makarov et al., 2010; Lucchini et al., 2019), investigations into HRV during the initial hours after birth are lacking. These studies have mostly recorded only a few minutes of ECG during the first day or start only beyond 12 h of age and none have described continuous HRV trends during the first 24 h of life.

Nonetheless, for some conditions, particularly those occurring due to birth complications and requiring time-sensitive decisions, such as neonatal encephalopathy, it is important to describe normal HRV reference values immediately after birth and their trends throughout the first 24 h. Such early trends may provide valuable information on how a baby has recovered from any birth-related complication. Our primary aim was to describe standard reference values for HRV trends over the first 24 h of postnatal life in healthy term infants. As a secondary aim, we investigated which (if any) clinical characteristics or risk-factors exert higher impact on HRV.

MATERIALS AND METHODS

Study Population

We prospectively and consecutively recruited 150 healthy term babies from the birth center, labor ward or postnatal ward at Queen Charlotte's and Chelsea Hospital between August 2017 and January 2019. We included healthy babies born at 36 weeks gestational age or more, following uncomplicated pregnancies, who were born in good condition with a birth weight between the 9th and 91st centile. Babies were excluded if they required any medication or phototherapy, if there was perinatal maternal pyrexia during or within 48 h of the onset of labor, if they required resuscitation at or after birth (intubation or cardiac compressions or any drugs) or if there was any intrapartum complication (maternal hemorrhage, placental abruptio, pre-eclampsia or cord prolapse). Our study only included babies who were well at birth and, therefore, stayed with their mothers at all times.

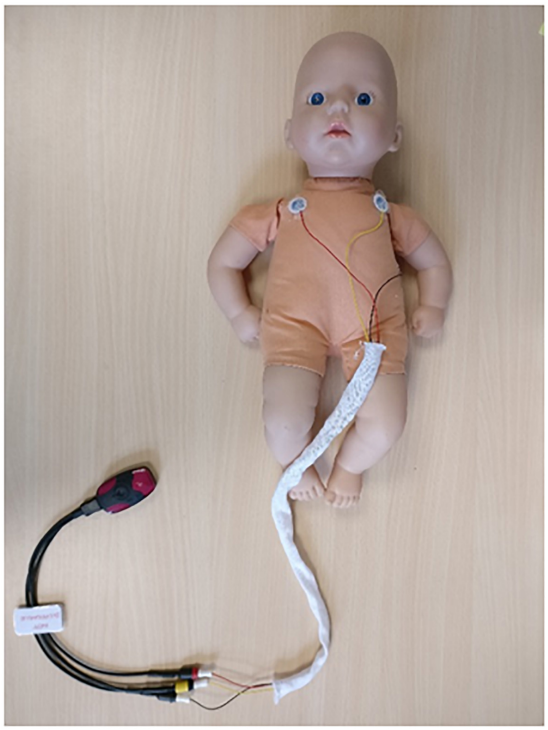

FIGURE 1 | Portable ECG recorder and thoracic setup. Three ECG electrodes were fed through an alignment sleeve to reduce the distance between ECG wires and prevent magnetic induction artifacts on raw ECG signal (500 Hz with automatic R-peak detection).

Intrapartum and Early Postnatal Care

Babies included in our study were born either at the birth center or in the labor ward, based on maternal preference. Women who preferred a more natural, less medicalized birth opted for midwife-led care in the birth center. A warm pool, aromatherapy, music, nitrous oxide and various equipment are available to help these women cope with the pain of labor. Women who opted for epidural analgesia received obstetrician-led care in the labor ward. In both environments, the room temperature was set at 24–25°C. As per national guidelines, babies born in good condition are given to the mother straight after birth and placed on their chest/abdomen for skin to skin care. They can be cleaned gently while on mother's chest, and breast or bottle feeding initiated within 1 h of birth.

ECG Acquisition

Electrocardiogram recordings were started as soon as possible after birth, following parental informed written consent, which could be obtained antenatally or postnatally. This study was approved by a National Research Ethics Committee (REC17/LO/0956) and by the local Research & Development department. Recordings were continued for at least 6 h but could be interrupted earlier if requested by the parents or if the baby was being discharged. We used a portable 2-inch ECG recorder (Faros 180, Bittium, Oulu, Finland) with triple-electrode thoracic setup and a sampling rate of 500 Hz (**Figure 1**), which we had previously tested. Once the recording was completed, the ECG file was uploaded onto Cardiscope^{TM} HRV Analysis Software (Hasiba Medical, Graz, Austria) for ECG and HRV analysis.

HRV Analysis

Heart rate variability metrics in the time (linear and non-linear) and frequency domains were calculated for each 5-min segment of the ECG (non-overlapping windows). We used a minimum QRS validity of 90%, which means all 5-min segments with less than 90% consecutive good quality QRS were excluded from analysis. Given the lack of international recommendations for HRV analysis specifically for neonates, our methods and chosen metrics were based on an adaptation of the available international guidelines (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Schwartz et al., 2002) plus recent recommendations for HRV research (Laborde et al., 2017) and a review of relevant HRV studies in neonates during the first days of life (Doyle et al., 2009; Goulding et al., 2015; Temko et al., 2015).

TABLE 1 | HRV metrics: abbreviations and meaning.

Metric	Definition
HR	Heart rate (number of heart beats per minute)
Time domain	
SDNN	Standard deviation of the consecutive RR intervals
RMSSD	Root mean square of the consecutive RR differences
SDSD	Standard deviation of consecutive RR differences
(∗) pNN20	Proportion of NN intervals that differ from the previous interval by at least 20 ms
(∗) pNN50	Proportion of NN intervals that differ from the previous interval by at least 50 ms
Geometric/Non-linear	
SD1	Standard deviation of the Poincaré cross-wise
SD2	Standard deviation of the Poincaré lengthwise
CVI	Cardiac Vagal Index = log (SD1 × SD2)
CSI	Cardiac Sympathetic Index = SD1/SD2
TINN	Triangular Index of the NN intervals – the length of the basis of the minimum square difference of the triangular interpolation for the highest value of the RR histogram or the normalized width of the base of the RR histogram.
HRV Index	Number of all RR intervals divided by the number of RR intervals at the highest point of the RR histogram
Parseval Index	Ratio between the square root of the sum of LF and HF powers and the value of SDNN
Frequency domain	
HFn	Normalized power in the high frequency band of the ECG spectrogram (0.20–2.00 Hz), i.e., high frequency power in relation to total power
LFn	Normalized power in the low frequency band on the ECG spectrogram (0.04–0.20 Hz), i.e., low frequency power in relation to total power
Total power	Total power of the ECG spectrogram

(*) pNN50 has been widely used in adults but may be less relevant in neonates given their higher heart rate. Hence, we have also reported pNN20 to adjust for neonatal heart rate which corresponds to a 4–5% variation, in a heart rate of 120–160 bpm.

TABLE 2 | Sample characteristics I (continuous variables).

	Mean(*SD*)	Min	Max
Gestational age (weeks)	38.7 (1.26)	36	42
Birth weight (grams)	3267.6 (461.62)	2080	4670
Gravida	2.83 (1.65)	1	8
Para	1.29 (1.19)	0	7
Cord pH	7.26 (0.08)	6.99	7.50
Cord BE	−3.61 (3.44)	−15.0	2.2

Gravida, number of pregnancies (including current); Para, number of previous live births; BE, base excess; Std, standard deviation; Min, minimum; Max, maximum.

TABLE 3 | Sample characteristics II (categorical variables).

Variable	Category	Frequency(%)
Single pregnancy	Yes	139 (92.7%)
	No	11 (7.3%)
TORCHS/Serology positive	Yes	3 (2%)
	No	147 (98%)
Smoking during pregnancy	Yes	2 (1.3%)
	No	148 (98.7%)
Alcohol during pregnancy	Yes	2 (1.3%)
	No	148 (98.7%)
Drugs during pregnancy	Yes	1 (0.7%)
	No	149 (99.3%)
Maternal depression	Yes	6 (4%)
	No	144 (96%)
Pregnancy induced or chronic	Yes	46 (30.7%)
maternal illness	No	104 (69.3%)
	Diabetes	22 (14.7%)
	Cardiac disease	0 (0.0%)
	Thyroid disease Epilepsy	6 (4%) 1 (0.7%)
	Hypertension	3 (2%)
	Other (*)	20 (13.3%)
Medications during pregnancy	Yes	25 (17%)
	Unknown	5 (3%)
	No	120 (80%)
	Antibiotics	2 (1.3%)
	LMW heparin	2 (1.3%)
	Other (**)	23 (15.3%)
Reduced fetal movements	Yes	12 (8%)
	No	138 (92%)
Cardiotocography	Not done	4 (2.7%)
	Unknown	1 (0.7%)
	Normal	132 (88%)
	Late deceleration	2 (1.3%)
	Bradycardia	2 (1.3%)
	Tachycardia	2 (1.3%)
	Variable deceleration	4 (2.7%)
	Other	3 (2.0%)
Analgesia during labor	No analgesia	9 (6%)
	50% Nitrous Oxide	40 (26.7%)
	General anesthesia	0 (0%)
	Spinal	1 (0.7%)
	Epidural	15 (10%)
	Combined Spinal and Epidural	86 (57.3%)
Labor onset	Spontaneous	53 (35.3%)
	Induced	28 (18.7%)
	Cesarean section (not in labor)	69 (46%)
Delivery mode	SVD	60 (40%)
	Forceps	2 (1.3%)
	Ventouse	12 (8%)
	Emergency Caesarean Section (not in labor)	1 (0.7%)
	Emergency Caesarean Section (in labor)	4 (2.7%)
	Elective Caesarean Section	71 (47.3%)
Labor and delivery events	No events	138 (92%)
	Antepartum hemorrhage	0 (0%)
	Shoulder dystocia	1 (0.7%)
	Meconium	11 (7.3%)
	Cord prolapse	0 (0%)
	Head entrapment	0 (0%)
	Other (***)	2 (1.3%)
Any resuscitation at birth	No	145 (96.7%)
	Yes	5 (3.3%)
	Stimulation	2 (1.3%)
	Suction	2 (1.3%)
	Bag and mask/Intermitent positive pressure ventilation (IPPV)	1 (0.7%)
Apgar scores [Med (IQR)]	At 1 min	9 (0)
	At 5 min	10 (0)
	At 20 min	10 (0)
Feeding method during recording period	Breastfeeding	125 (83.3%)
	Bottle-feeding	25 (16.7%)

TORCH – Toxoplasmosis, Rubella, Cytomegalovirus, Herpes simplex. Serology refers to any other available serology tests including Human Immunodeficiency Virus (HIV), Hepatitis B/C and Syphilis. (*) Other pregnancy induced or chronic maternal illness: Asthma (7); Crohn's disease (1); Hemophilia (1); Latent tuberculosis (1); Infertility problems (1); Group B streptococcus (1); Graves' disease (1); Thalassemia carrier (1); Obstetric cholestasis (1); Hashimoto's disease (2); Polycystic ovarian syndrome; Sickle cell carrier (1); Osteoporosis (1); Arthritis (1). (**) Other Medications during pregnancy: Metformin (11); Dihydrocodeine (1); Insulin (2); Propranolol (1); Levothyroxin (4); Tinzaparin (1); Citalopram (1); Ursodeoxicolic acid (1); Levatiracetam (1) Amitriptyline (1); Salbutamol (2). (***) Other labor and delivery events: Circular cervical cord (1); Prolonged 2nd stage of labor (1).

The 16 HRV metrics we chose to analyze were based on these references. Frequency analysis was done with Fourier transform (Welch Periodogram) and we used detrended and interpolated (cubic spline) RR interval time series. Based on the above literature, we used a LF band of 0.04–0.20 Hz and a HF band of 0.20–2.0 Hz. We then analyzed normalized LF and HF, i.e., the proportion of power in those ranges in relation to the total spectral power. The list of HRV metrics reported in this study and their meaning are described in **Table 1**.

Statistical Analysis

We used Stata 15 (StataCorp, Austin, TX, United States) for the statistical analysis. We described HRV time trends with hourly medians and inter-quartile ranges and calculated the individual averages for the first 6 and 24 h of life. Since the ECG recordings could start and end at different times, our data was imbalanced, i.e., we did not have the exact same number of measurements for all participants, changes in HRV over time were analyzed with multi-level mixed effects regression with autoregressive covariance or using pairwise tests if comparing six hourly averages. The relevance of clinical variables was also tested with multi-level mixed effects regression with autoregressive covariance and subgroup comparisons were performed using proportions/mean comparison-tests for the significant clinical variables. As most trends were not linear and had at least one deflexion, we used a quadratic term for the time variable in the regression model. We log-transformed HRV variables which were not normally distributed to ensure normal residuals.

RESULTS

Between September 2017 and January 2019, we screened 511 babies of which 360 were eligible and 151 were not. Out of those 360 babies, 201 mothers/fathers requested us to return later or at another convenient time, which eventually surpassed the maximum recruitment window or became impossible to recruit due to equipment being in use. Out of 159 mothers/fathers who were fully informed about the study, 9 declined and 150 gave informed written consent. Of these 150 participants, 7 started ECG recording after 24 h. Sample characteristics are reported in **Tables 2**, **3**. In total, we obtained 1858 h and 55 min of ECG recording starting at a median (IQR) age of 2 h 46 min (3 h 6 min), minimum 1 min after birth, maximum 52 h 23 min. Not all babies started ECG recording at the same time nor did all recordings last the same duration. **Figure 2** presents the number of valid recordings and babies by time.

HRV Values Over Time

During the first 6 h of postnatal age, median (IQR) values were HR 122 (15.9), SDNN 27.5 (13.2), RMSSD 18.32 (11.42), SD1 13.6 (7.7), SD2 36.2 (17.8), SDSD 18.8 (11.4), CVI 2.7 (0.4), CSI 2.6 (1.2), pNN20 14.8 (15.2), pNN50 1.7 (2.5) HFn 40.4 (16.9) LFn 57.7 (17.8) Total power 751 (835), TINN 226 (144) HRV Index 5.9 (2.3) Parseval 0.7 (0.2). Nine of these HRV metrics (including heart rate) changed significantly over time (HR $p < 0.01$; SDNN $p = 0.01$; SD2 $p < 0.01$; CSI $p < 0.01$; HFn $p = 0.03$; LFn $p < 0.01$;Total power $p < 0.01$; HRV Index $p = 0.01$; Parseval Index $p = 0.03$), adjusted for relevant clinical variables. A more pronounced variation was observed during the first 6 h of postnatal life. Only heart rate and LFn changed between 6 and 12 h of age and only heart rate changed between 12 and 18 h of age (Bonferroni adjusted p-values: 0.03, < 0.01 and < 0.01, respectively). These metrics exhibited increasing HRV during the first 6 h followed by a slight decrease up to 12 h from which point HRV remained stable (**Table 4**). Hourly trends expressed by median and interquartile ranges are presented in **Figure 3**. HRV trends over time were independently affected by gestational age [7 metrics: RMSSD ($p = 0.01$), SDSD ($p = 0.01$), SD1 ($p = 0.01$), CVI ($p = 0.02$), pNN20 ($p = 0.03$), TINN ($p = 0.02$), Parseval Index ($p = 0.01$)], reduced fetal movements [7 metrics: RMSSD ($p = 0.01$), SDSD ($p = 0.01$), SD1 ($p = 0.01$), CSI ($p = 0.01$), pNN20 ($p = 0.01$), pNN50 ($p = 0.01$), Parseval Index ($p = 0.03$)], cardiotocography (CTG) classification [3 metrics: CSI ($p = 0.03$), LFn ($p = 0.01$), HFn ($p = 0.02$)], maternal chronic or pregnancy induced illness [2 metrics: CSI ($p = 0.02$), Parseval Index ($p = 0.01$)] and occurrence of delivery complications [2 metrics: heart rate ($p = 0.01$) and CSI ($p = 0.04$)]. We examined the interdependencies of all HRV metrics with a correlation matrix (**Figure 4**), where we highlight (a) the similarity between time-domain metrics and (b) the trend toward symmetry between HFn and LFn and CSI and CVI.

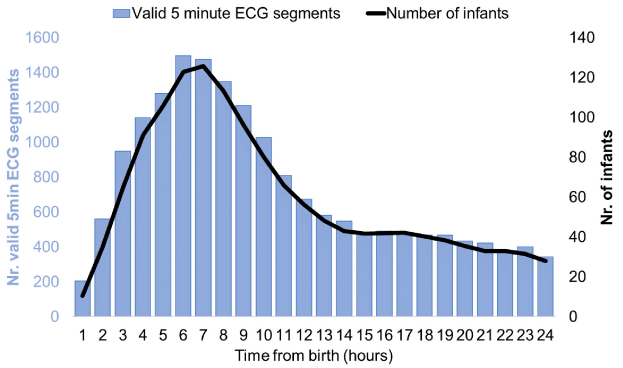

FIGURE 2 | Recording validity and sample size by time.

Effect of Clinical Factors

In addition to age (time form birth), univariable multi-level mixed effects regression showed that HRV metrics were affected by gestational age, reduced fetal movements, CTG, maternal illness and delivery complications. After adjusting for these variables, the variation over time of the above seven HRV metrics remained statistically significant. HFn and Parseval Index also showed significant changes. Although parity was not an independent predictor on univariable regression analysis, in the subgroup of spontaneous vaginal deliveries, we observed a consistent trend toward higher HRV for primiparas in relation to multiparas.

Reduced Fetal Movements

Reduced fetal movements were consistently associated with lower HRV values for all the metrics except for CSI (which behaves in the opposite direction, i.e., the result is concordant). Such differences between subgroups were only statistically significant for CSI ($p = 0.001$), pNN20 ($p = 0.045$) and Parseval Index ($p = 0.047$) trends. **Figure 5** presents the trends of these three metrics over time, in comparison to heart rate. This is despite no significant differences between sub-groups, other than average gravidity, which did not

TABLE 4 | Six-hourly HRV averages and pairwise comparisons.

						Overall	Pairwise comparison		
HRV Metric		**6 h**	**12 h**	**18 h**	**24 h**	**24 h trend**	**6 vs. 12 h**	**12 vs. 18 h**	**18 vs. 24 h**
HR	Mean	124.05	121.66	125.92	130.47	$p < 0.01$	0.01	0.0018	0.25
	SD	0.96	0.83	1.35	1.16				
SDNN	Mean	29.81	29.56	27.32	26.45	$p = 0.01$	0.72	0.97	0.87
	SD	0.87	0.92	1.02	1.04				
RMSSD	Mean	21.78	21.08	19.18	18.58	$p = 0.18$	0.42	0.83	0.46
	SD	0.84	0.8	1.19	1.39				
SD1	Mean	15.4	14.9	13.56	13.14	$p = 0.18$	0.42	0.82	0.46
	SD	0.59	0.56	0.84	0.98				
SD2	Mean	38.48	38.41	35.44	34.39	$p < 0.01$	0.49	0.95	0.06
	SD	1.15	1.23	1.28	1.27				
SDSD	Mean	21.77	21.08	19.18	18.58	$p = 0.18$	0.42	0.82	0.46
	SD	0.84	0.8	1.19	1.39				
CVI	Mean	2.69	2.68	2.61	2.6	$p = 0.31$	0.07	0.93	0.47
	SD	0.03	0.03	0.04	0.04				
CSI	Mean	2.72	2.8	2.96	2.96	$p < 0.01$	0.10	0.90	0.72
	SD	0.07	0.07	0.14	0.14				
pNN20	Mean	18.33	19.66	16.21	15.01	$p = 0.07$	0.06	0.51	0.49
	SD	0.96	1.03	1.59	1.81				
pNN50	Mean	3.36	2.88	2.38	2.5	$p = 0.31$	0.23	0.25	0.94
	SD	0.4	0.31	0.37	0.79				
HFn	Mean	40.19	40.84	41.19	40.56	$p = 0.03$	0.67	0.90	0.77
	SD	1.1	1	1.8	1.68				
LFn	Mean	55.15	59.15	58.81	59.29	$p < 0.01$	0.0004	0.90	0.77
	SD	1.17	1	1.8	1.64				
Total p.	Mean	1098.2	1073	884.85	831.68	$p < 0.01$	0.31	0.69	0.34
	SD	94.43	81.96	78.09	76.98				
TINN	Mean	259.32	254.06	232.16	228.29	$p = 0.27$	0.88	0.84	0.17
	SD	8.94	9.35	15.4	1463				
HRV Index	Mean	6.17	6.2	6.01	5.95	$p = 0.01$	0.53	0.55	0.74
	SD	0.14	0.17	0.21	0.21				
Parseval I.	Mean	0.66	0.7	0.67	0.68	$p = 0.03$	0.04	0.99	0.09
	SD	0.01	0.01	0.02	0.02				

Overall trend p-values result from multilevel mixed effects regression analysis, adjusted to all relevant clinical variables. Pairwise comparison p-values displayed are uncorrected for multiple comparisons. Bonferroni-corrected p-values for significant results are reported in the main body of text in the results section. For abbreviations of HRV metrics, please refer to ***Table 1****.*

independently predict HRV (**Table 5**). The associations with CSI and pNN20 remained significant after adjusting for gestational age, time from birth, CTG classification, presence of maternal chronic illness or delivery complications (**Table 6**). The subgroup with reduced fetal movements had lower average HRV during the first 6 h, although these differences were not statistically significant.

Cardiotocography

There was a significant association between CTG findings and HRV trends over time for CSI (p = 0.03), LFn (0.01), and HFn (p = 0.02), which remained statistically significant after adjusting for reduced fetal movements, gestational age, time from birth, presence of chronic maternal or pregnancy induced illness or delivery complications. Nonetheless, given the small number of events in each CTG classification we also compared the 6 h mean across these subgroups. CSI in the bradycardia subgroup was significantly different from the "normal," "variable deceleration," and "other" groups (Bonferroni adjusted p-values: 0.02, 0.03, and 0.04, respectively) but no other group differences were statistically significant. HFn was only different between "bradycardia" and "variable deceleration" subgroups (p = 0.03) and LFn did not vary between different CTG subgroups.

Maternal Chronic or Pregnancy Acquired Illness

In our sample, 104 (69%) women had no chronic illness nor pregnancy induced disease, 20 (13%) had isolated diabetes mellitus or gestational diabetes, 7 (3%) had thyroid disease, 1 (0.7%) had hypertension and the remaining [22 (15%)] had other conditions, including combinations of the above (**Table 3**). Maternal chronic or pregnancy induced illness

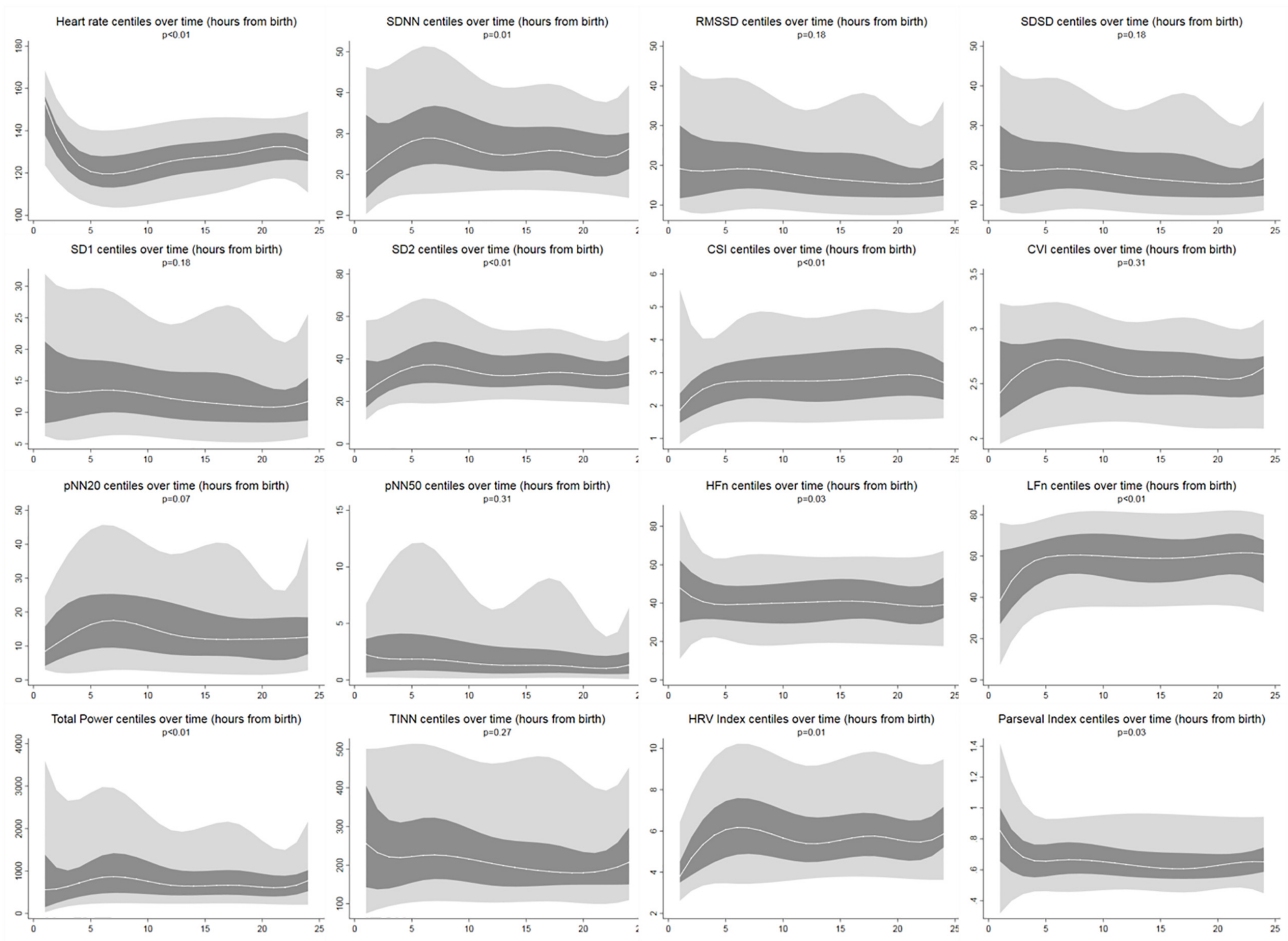

FIGURE 3 | HRV centiles during the first 24 h of age. Light gray areas: 5th–25th centiles and 75th–95th centiles. Dark gray areas: 25th–75th centiles. Middle white line corresponds to the median. Reported *p*-values result from multi-level mixed effects regression to measure change over time [autoregressive covariance (time), repeated measures (id)]. All centiles smoothed with cubic spline.

was significantly associated with unadjusted HRV trends [CSI (p = 0.02) and Parseval Index (p = 0.01)] although this only remained statistically significant for Parseval Index (p = 0.03) after adjusting for clinical confounders. On binary analysis, babies of mothers with pregnancy/chronic illness did not have different average Parseval Indexes either during the first 6 h (p = 0.98) or during the 24 h period (p = 0.29). The disease group with lowest Parseval Index was thyroid disease.

Events During Labor and Delivery

All babies in our study were born following uncomplicated pregnancies and deliveries and born in good condition. Nonetheless, there were 13 births that had one of the following events: meconium stained liquor (11), circular cervical cord (1), prolonged 2nd stage of labor (1) and shoulder dystocia (1, in addition to meconium). CSI trends over 24 h were significantly associated with presence of any labor and delivery events before (p = 0.04) but not after adjusting for confounders (p = 0.42).

Although delivery mode did not independently predict HRV values, babies born via instrumental delivery had lower 24 h HFn and higher 24 h CSI [mean (95% CI) 36.7 (34.0–39.4) vs. 42.3 (41.2–43.3) and 3.1 (2.9–3.3) vs. 2.7 (2.7–2.8), respectively, p = 0.002] than babies born via natural vaginal delivery. This was despite no significant differences between other relevant clinical variables, except Apgar Score at 1-min and multiplicity of gestation which did not independently predict HRV (**Table 7**).

DISCUSSION

This is the first study to describe early postnatal continuous HRV trends in healthy term babies in the immediate postnatal period. Identifying these thresholds and trends was important because we now know that HRV analysis and interpretation in the early postnatal period is time-dependent, i.e., what is normal at 1–6 h of age may not be normal at 12–18 h of age. This will allow clinicians and researchers to more accurately examine the differences in HRV between healthy and sick infants

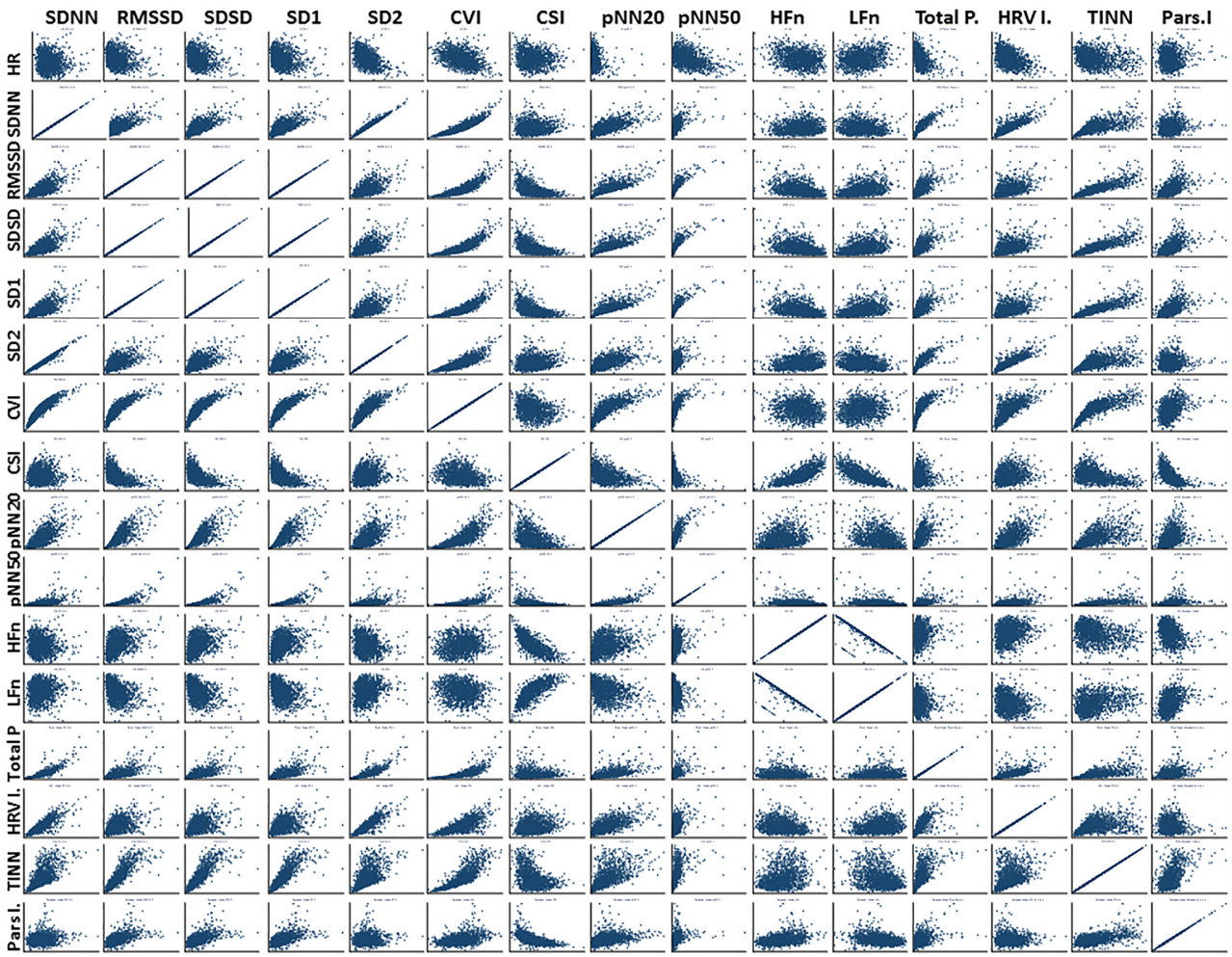

FIGURE 4 | Correlation matrix: all HRV metrics. For abbreviations of HRV metrics, please refer to **Table 1**. We note particularly (a) the concordance between RMSSD, SDSD, and SD1, which has been previously described; (b) the behavior of different HRV metrics in relation to HR which highlights how much additional information HRV analysis can provide; (c) the interdependency of HFn and LFn and CVI and CSI, highlighting the interplay between sympathetic and parasympathetic influences.

in the immediate postnatal period. Having accurate reference values for the immediate postnatal period also means that we are now better equipped to develop early warning systems based on HRV analysis.

There are some possible reasons for the changes we observed in the first 6 h after birth. Since birth has been previously described as a stressful event for babies (Peebles et al., 1994; Aldrich et al., 1996), it is possible that the improving HRV in the first hours reflects the end of the stressor effect (i.e., the end of birth). This parasympathetic rebound could occur because the sympathetic nervous system can temporarily supress parasympathetic activity which stops once the stress period is over. On a different perspective, Reyes-Lagos et al., 2015 reported higher maternal HRV during labor than during the third trimester and Musa et al., 2017 described increasing LFn and HFn by cervical dilation during labor. If fetal HRV follows maternal HRV, this would instead suggest that birth may represent a period of particularly high HRV reflecting the good adaptation to physiological challenges, in healthy babies. Finally, it is also possible that the changes in HRV observed during the first hours are a partial expression of the HRV maturation that occurs with age (Fyfe et al., 2015).

We have reviewed HRV findings reported in other neonatal and fetal studies with the intent of comparing such values with those in our study but given the differences in metrics and acquisition and duration for recordings, this was very challenging. The HRV values we observed in the first 6–12 h were comparable (slightly higher) to those reported by Doyle et al. (2009) in active sleep during the first 12 h of life and Lucchini et al., 2019 at 12–84-hour-old and lower than those reported at older ages, i.e., 24–168 h (by Mehta et al., 2002; Longin et al., 2005; Makarov et al., 2010). Babies in our study had SDNN values during the first 6 h of age that were comparable (slightly higher) to those in term fetuses (Brändle et al., 2015; Schneider et al., 2018), which was expected, given the

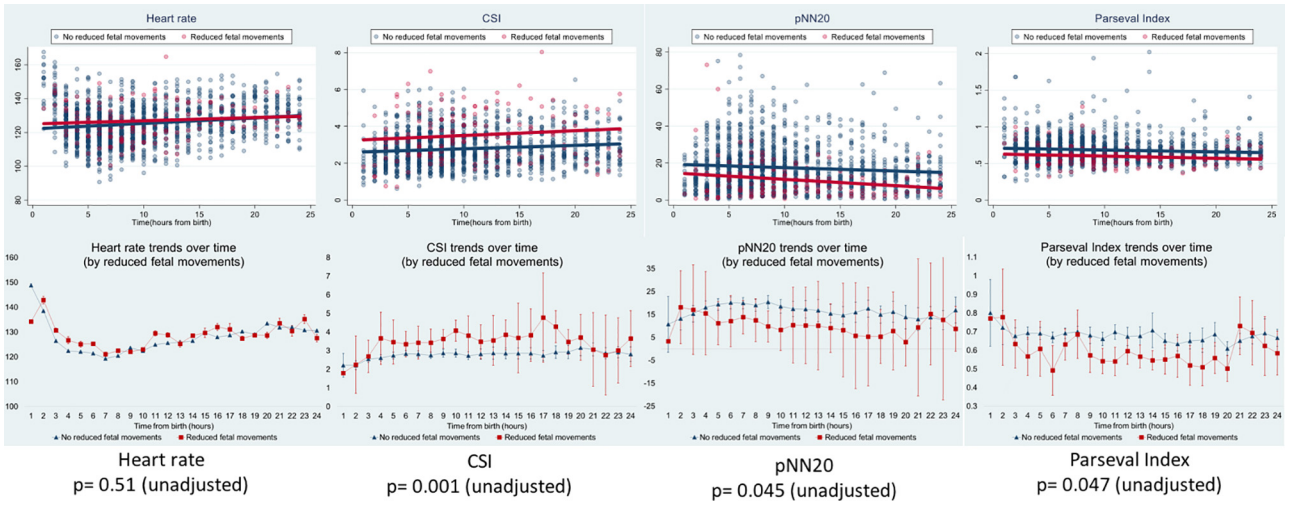

FIGURE 5 | HRV changes in babies whose mothers reported reduced fetal movements. For abbreviations of HRV metrics, please refer to **Table 1**. Upper panels present HRV data scatter over time and linear trend lines for heart rate (comparative) and the three HRV metrics that were significantly associated with maternal reports of reduced fetal movements. Red lines represent babies whose mothers reported reduced fetal movements and blue lines represent babies whose mothers did not. The lower panels present the means and 95% confidence intervals for those metrics.

TABLE 5 | Subgroup comparison – babies with and without reduced fetal movements.

Clinical variable	No reduced fetal movements (*n* = 136)	Reduced fetal movements (*n* = 14)	*p*-value
Gestational age (weeks)	38.68	38.86	0.70
Birth weight (grams)	3266.47	3278.57	0.92
Cord base excess	−3.53	−4.14	0.61
Cord pH	7.26	7.25	0.71
Gravida	2.93	1.93	0.01
Para	1.34	0.86	0.16
Apgar 1 min	9 (0)	9 (0)	0.13
Apgar 5 min	10 (0)	10 (0)	0.18
Multiple Pregnancy	10 (7.3%)	1 (7.1%)	0.73
Positive TORCHS/Serology	4 (2.9%)	0 (0%)	0.67
Maternal smoking	2 (1.5%)	0 (0%)	0.82
Maternal alcohol use	2 (1.5%)	0 (0%)	0.82
Maternal drug use	1 (0.7%)	0 (0%)	0.91
Maternal depression	6 (4.4%)	0 (0%)	0.55

Values presented are in means for variables Gestational age to Para; medians (inter-quartile ranges) for Apgar scores and proportions (percentages) for remaining variables. Gravida – number of previous pregnancies including the one leading to current baby. Para – number of previous births. TORCH – Toxoplasmosis, Rubella, cytomegalovirus, Herpes simplex. Serology refers to any other available serology tests including Human Immunodeficiency Virus (HIV), Hepatitis B/C, and Syphilis. P-values derived from unequal variances t-test for continuous variables and Fisher's exact test for binary variables.

longer recording duration and increasing gestational age. Having similar HRV before and after birth supports the theory that birth, in healthy babies, is associated with good HRV stability.

We observed more inter-subject than within-subject variability across all HRV metrics. This emphasizes that HRV analysis must be interpreted based not only on reference values but also take into consideration the changes and progress in relation to individual baselines. In fact, whereas we could observe hourly HRV changes during the first 6 h of age, we might not have been able to detect such variation if we had only committed to analyze a single time-point or average. Monitoring such trends may provide further insight about how an infant recovers from birth in the event of a complicated delivery or emergency intervention. Indeed, the fact that HRV metrics were significantly associated with reduced fetal movements, abnormal CTG, maternal chronic or pregnancy induced illness and delivery complications highlights the value of HRV analysis as a measure of overall wellbeing.

The association between HRV and fetal movements has been previously reported by Brändle et al. (2015) using fetal biomagnetography. In their study assessing behavior state based on movements which included healthy fetuses from 24 to 41 weeks, HRV metrics (but not Entropy) were increasing from quiet sleep to active sleep and from active sleep to awake state, for all gestational ages. In fact, Nijhuis et al. (1982) had already proposed a fetal movement classification based on fetal HRV, eye movement and body movement.

Previous authors have found differences in HRV values across different delivery modes. Kozar et al. (2018) reported lower HFn and higher LFn in babies born via cesarean section than in babies born vaginally. It is possible that their finding is related with the use of Thiopental for general anesthesia (GA) in all their elective sections (Riznyk et al., 2005; Tsuchiya et al., 2006) rather than with the delivery mode, whereas in our study there was no use of any GA. Our interpretation is that HRV will indicate a difference between delivery modes if there is a difference in wellbeing, therefore, in our study, this would have

TABLE 6 | Unadjusted and adjusted correlation between HRV metrics and reduced fetal movements.

	Unadjusted					Adjusted				
	Coef.	***SD***	***p***	**95% CI**		**Coef.**	***SD***	***p***	**95% CI**	
CSI	0.66	0.21	0.011	0.26	1.07	0.65	0.22	0.003	0.22	1.08
pNN20	−5.96	2.98	0.045	−11.8	−0.13	−7.53	3.24	0.02	−13.9	−1.17
Parseval	−0.07	0.36	0.047	−0.14	−0.00	−0.08	0.4	0.054	−0.15	0.00

*For abbreviations of HRV metrics, please refer to **Table 1**. Analysis based on multi-level mixed effects regression analysis (auto-regressive covariance). Adjusted results based on including all clinical variables that were significant predictors on univariable analysis, i.e., gestational age, time from birth, CTG, maternal illness, and delivery complications.*

TABLE 7 | Subgroup comparison – babies born via normal vaginal versus instrumental delivery.

Clinical variable	**Spontaneous vaginal delivery (*n* = 60)**	**Instrumental delivery (*n* = 14)**	***p*-value**
Gestational age (weeks)	38.77	37.89	0.45
Birth weight (grams)	3242.69	2955.56	0.39
Cord base excess	−4.54	−5.57	0.47
Cord pH	7.25	7.20	0.06
Gravida	3.27	2.11	0.39
Para	1.69	0.55	0.08
Apgar 1 min	9 (0)	9 (1)	0.03
Apgar 5 min	10 (0)	10 (0)	0.52
Reduced fetal movements	6 (10%)	3 (21.4%)	0.22
Multiple Pregnancy	0 (0%)	2 (14.3%)	0.03
Positive TORCHS/Serology	0 (0%)	0 (0%)	Not applicable
Maternal smoking	1 (1.7%)	0 (0%)	0.81
Maternal alcohol use	0 (0%)	0 (0%)	Not applicable
Maternal drug use	0 (0%)	1 (7.1%)	0.19
Maternal depression	2 (3.3%)	2 (14.3%)	0.16

Values presented are in means for variables Gestational age to Para; medians (inter-quartile ranges) for Apgar scores and proportions (percentages) for remaining variables. Gravida – number of previous pregnancies including the one leading to current baby. Para – number of previous births. TORCH – Toxoplasmosis, Rubella, cytomegalovirus, Herpes simplex. Serology refers to any other available serology tests including Human Immunodeficiency Virus (HIV), Hepatitis B/C, and Syphilis. P-values derived from unequal variances t-test for continuous variables, Krushkal–Wallis for non-parametric variables and Fisher's exact test for binary variables.

been associated with the use of instruments during delivery due to difficult extraction.

Limitations

We have not examined the sleep states of the recruited infants during the first 24 h after birth. It is unlikely that babies would have established a circadian within few hours of birth and often newborn babies follow ultradian rhythms instead (Mirmiran et al., 2003). Nevertheless, it is possible that the slightly downward trend that we observed in the second half of the 24 h recordings reflects a bigger proportion of babies sleeping or in a quieter state. We have included a few healthy babies in our study whose mothers had some chronic or pregnancy induced illnesses. It could be argued that including these cases compromises our definition of healthy newborn and healthy neonatal HRV. The fact that there were no significant associations between any HRV metric and pH or base excess or Apgar score highlights that our sample was indeed healthy, as all the babies in our study were born in good condition and did not require any type of investigations or treatment. Our aim was to represent the whole spectrum of deliveries that are considered and clinically managed as "healthy" from a pragmatic point of view (i.e., all "low-risk" and "uncomplicated"). Thus, including those babies was an important step in addressing possible "variations of normal" and to enrich our dataset. Equally, we have explored possible differences in HRV across several subgroups (according to clinical variables such as described in **Table 2**). It is important to clarify that our study was not aimed at or powered to address in detail possible differences across those subgroups and caution is needed to interpret our findings. Nonetheless, we believe that examining these associations will be of interest for healthcare teams in obstetric and perinatal/neonatal care.

CONCLUSION

We have described the early postnatal reference values for HRV metrics in healthy term infants, which had not been previously achieved. HRV changes significantly during the first day of life, particularly during the first 6 h, during which it seems to exhibit a brief increase followed by normalization. The significant correlations between HRV and clinical risk variables support the hypothesis that HRV is a good indicator of overall wellbeing of a baby and is sensitive to pick up birth-related stress and monitor its resolution over time.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the United Kingdom Health Research Authority and the GCP ICH with written informed consent

from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the London Chelsea Research Ethics Committee.

AUTHOR CONTRIBUTIONS

VO designed the study, collected, analyzed, and interpreted the data, wrote the first draft and led the development of the manuscript. WvR contributed to the analysis and interpretation of the data and provided critical inputs for developing the manuscript. PM and TA contributed to the interpretation of the data and provided critical inputs for developing the manuscript. JM contributed to the study design and data interpretation, recruited patients and acquired data. VS contributed to the study management and recruitment. DM supervised all aspects of HRV analysis and interpretation and provided critical inputs for developing the manuscript. ST conceived the idea and designed the study, supervised all aspects of the study and led the development of the manuscript.

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Infants Autonomic Cardio-Respiratory Responses to Nurturing Stroking Touch Delivered by the Mother or the Father

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The building of physiological self-regulation during bonding is a crucial developmental process based on early cardio-respiratory maturation. The mother's role as a facilitator of this physiological maturation has been evidenced and recognized in many respects. Research in fathers, however, remains sparse which may be due to the belief that bonding is a physiological behavior reserved for a mother's maternal instinct. In the current study we compared the impact of paternal and maternal nurturing stroking touch on infants' physiological self-regulation in terms of respiratory sinus arrhythmia (RSA). We compared the impact of a 3-min stroking period (STROKING) with a pre-baseline (PRE-STROKING) and post-baseline (POST-STROKING) of 25 mothers and 25 fathers (unrelated to one another) on their infants, aged 4–16 weeks. We registered infant electrocardiogram (ECG) and respiration to calculate infant RR-interval (RRI), respiration rate (*f*R) and (respiratory corrected) RSA (RSA*corr*). Based on video-recordings, we analyzed the stroking speed. Infants' RSA*corr* significantly increased during and after stroking, no matter whether touch was delivered by fathers or mothers. This effect was mediated by both heart rate (HR) and respiration. However, respiratory mediation occurred later when delivered by fathers than by mothers. Both mothers' and fathers' stroking speed occurred within the optimal stimulation range of c-tactile (CT) afferents, a particular class of cutaneous unmyelinated, low-threshold mechano-sensitive nerves hypothesized to be involved in inter-personal bonding. The discussion builds on the idea to mitigate fathers' doubts about their paternal capabilities and proposes a research agenda regarding the further examination of the role of nurturing touch and its underlying mechanisms within the development of infants' physiological self-regulation. Finally, the importance of respiratory measurements in infant physiological research is emphasized.

Keywords: maternal-infant touch, paternal-infant touch, stroking touch, c-tactile afferents, respiratory sinus arrhythmia

INTRODUCTION

Establishing physiological and emotional bonding during the 1st months of life paves the way for one's capacity to attach and to form intimate relationships later in life (Bowlby, 1969). This bonding process comes into being by the exchange of recurrent reciprocal cycles of sensitive responsive parent care and infant signals (Papoušek, 2007) which are known to form the source of further beneficial development. From a physiological and neuroendocrine perspective, the bonding period provides critical neuroendocrine and physiological exchanges that facilitate the development of a well-functioning stress-regulation system by epigenetic regulation of glucocorticoid receptor gene expression (e.g., Meaney, 2001; Curley and Champagne, 2016). From a social-emotional perspective, a fluent cycle of parenting is characterized by parent-infant contingency helping the infant to understand its social environment (Papoušek, 1984, 2007). When analyzing parent-infant interactions from the perspective of contingency, it was shown that fathers are able to interact with a comparable sensitive responsivity as mothers (e.g., Lynn and Sawrey, 1959; Hetherington, 1970; Biller, 1971, 1974; Block, 1973; Lynn, 1974; Maccoby and Jacklin, 1974; Sawin and Parke, 1979). Moreover, there is a growing tendency of fathers with regard to the amount of interaction with their infants. In the United Kingdom, father-infant engagement multiplied 8-fold between 1975 and 1999 (Smith, 2004) and interactions have been extended from averaging 15 min to 2 h per day (Fisher et al., 1999). As with mothers, the presence or absence of a father figure as well as the quality of father-infant interaction are playing a significant role in the development of an infant's social-emotional behavior (Black et al., 1999; Cummings et al., 2005; Ramchandani et al., 2005; Paulson et al., 2006; Shannon et al., 2006; Bretherton, 2010).

Nevertheless, when it comes to the bonding period and caregiving responsibilities in particular, fathers are still stigmatized as 'slow to warm up' and shy to act. Although fathers do express an increased desire to connect emotionally with their infant (Cheng et al., 2011; Lee et al., 2013; Johnson, 2017) there is a group that persevere in a 'gendered physiology-focused' bias (Brady et al., 2016). These fathers view infant bonding as a physiological-based behavior that is solely the province of the mother. They report feelings of uselessness during the infant's 1st months of life, not considering themselves being on the same plane as the mother who would have—in their opinion—the physiological advantage of having carried the infant during pregnancy and breastfeeding it postnatally (Brady et al., 2016). A foundation for this persisting bias can be found in the initial iteration of attachment theory (Bowlby, 1969). Before attachment became a multiple concept (e.g., Lamb, 1981), Bowlby (1969) sketched a monotropic parenting model with the mother as the only bonding and attachment figure for an infant.

Also in research, the mother's role has always been emphasized with the mother-infant dyad being the main focus of study in infant interaction and development. Certainly, with regard to the development of physiological self-regulation, the current insights are mainly based on mother-infant research. These studies have shown that during close body-contact, infants benefit from a transfer of mother-infant parasympathetic regulation which aids them to mature their self-regulatory capacities (e.g., Christensson et al., 1992; Bystrova et al., 2003; Winberg, 2005; Van Puyvelde et al., 2015) that still need to develop during the 1st year of life (Bar-Haim et al., 2000). This parasympathetic transfer between mothers and infants has been ascribed to the induction of parasympathetic vasodilatation, increased blood flow, temperature (Christensson et al., 1992; Bystrova et al., 2003) and in later studies to cardiorespiratory regulation (Bloch-Salisbury et al., 2014; Van Puyvelde et al., 2015). However, although the importance of a warm 'maternal nest' (Winberg, 2005, p. 218) in the transfer of overall parasympathetic regulation has indisputably been demonstrated (Christensson et al., 1992; Bystrova et al., 2003; Van Puyvelde et al., 2015), this does not mean that a warm 'paternal nest' could not bring about similar effects.

Parasympathetic regulation is reflected at a cardio-respiratory level (Thayer and Lane, 2000, 2009) by heart rate variability (HRV). Within HRV, respiratory sinus arrhythmia (RSA) reflects the specific component related to the parasympatho-inhibitory impact on the heart, mediated by the nervus vagus (i.e., cranial nerve X) (Berntson et al., 1997). RSA is obtained by measuring ECG and respiration and calculated by the peak-valley method based on a breath-by-breath analysis in coordination with the cardiac events. In the peak-valley method, the mean difference between the longest heart period during expiration and the shortest heart period during inspiration is calculated for each respiration cycle (Grossman et al., 1990). This method is preferred in infant research (Ritz et al., 2012; Van Puyvelde et al., 2014, 2015, 2019), because of infants' immature respiratory control (Rother et al., 1989). For instance, Ritz et al. (2012) and Van Puyvelde et al. (2015) reported that the inclusion of respiration revealed that in approximately 30% of the breaths a reliable RSA-value could not be detected which would had led to false positives at these moments.

Although research into the impact of paternal care on infants' physiological events are sparse, a significant amount of research has been done on hormone production in parents. In fathers specifically, active playing 'rough and tumble' interactions have been reported to be positively correlated with oxytocin (Feldman et al., 2010) and testosterone production (Rilling and Mascaro, 2017) whereas empathy-related caring interaction behavior would be negatively correlated with testosterone (Fleming et al., 2002; Mascaro et al., 2014; Weisman et al., 2014).

The associations between oxytocin levels and paternal touch are interesting with regard to the gendered physiology-focused bias in fathers reported by Brady et al. (2016). Gentle stroking has been shown to induce oxytocin release in animals (e.g., Uvnäs-Moberg et al., 1993; Winslow et al., 2003; Okabe et al., 2015) and human infants (Matthiesen et al., 2001) and to stimulate RSA in human infants (Van Puyvelde et al., 2019). One hypothesis with regard to potential underpinnings can be found in the activity of a relatively recently characterized population of mechanosensitive unmyelinated nerves called c-tactile afferents (CTs) (Vallbo et al., 1999; Löken et al., 2009). CT afferents are found in hairy skin areas of the body and are absent in the glabrous skin (Vallbo et al., 1999; Ackerley et al., 2014b) and are preferentially responsive to

gentle stroking touch within a 1–10 cm/s velocity range (Löken et al., 2009). In adults, this velocity, in comparison with slower and faster velocities, has been reported to be perceived as most pleasant (Essick et al., 1999, 2008; Löken et al., 2009). In mother-infant interaction it has been shown that mothers stroked their infants within a CT-afferent optimal stroking speed window and at CT-afferent rich body locations (Croy et al., 2016; Walker et al., 2017; Van Puyvelde et al., 2019). Moreover, with regard to the earlier comments on the importance of a 'warm nest' it is on interest from a neurobiological point of view that CTs are optimally responsive not just to the velocity of a nurturing touch, but also to its temperature (Ackerley et al., 2014a, 2018)—again attesting to the known value of skin-to-skin contact in regulating the infants' (Bystrova et al., 2003) as well as adults' (Holt-Lunstad et al., 2008) temperature.

To clarify the link between stroking touch and parasympathetic stimulation, Van Puyvelde et al. (2019) recently hypothesized that a CT afferent responsive brain network (i.e., the posterior insular cortex, mid-anterior orbitofrontal cortex, anterior cingulate cortex and amygdala) (Olausson et al., 2002; McGlone et al., 2012; Gordon et al., 2013) may overlap the zones responsible for neurovisceral integration and parasympathetic regulation in terms of RSA (Thayer and Lane, 2000, 2009). Indeed, on the level of the infant brain, gentle stroking touch evokes activity in the insular cortex of 4 weeks old infants (Tuulari et al., 2017) and 8 weeks old infants (Jönsson et al., 2018). Moreover, the insula is also involved in thermo-sensation making this modality as a submodality of touch (Craig et al., 2000). On a cardiorespiratory level, increased RSA is evoked by maternal stroking touch but not by non-stroking touch (Van Puyvelde et al., 2019) and a study by Fairhurst et al. (2014) showed decreased heart-rate responses in infants in a 10 s-window after optimal CT afferent stroking touch (i.e., a velocity of 3 cm/s but not after 0.3 or 30 cm/s).

In summary, nurturing touch has been indicated as one of the key-components in the development of self-regulatory capacities in infants (RSA), a physiological nurturing behavior that for certain fathers is reserved for a mother's maternal instinct (i.e., gendered physiology-focused bias). Research showed that only stroking affective touch, and not non-stroking affective touch provided RSA stimulation in infants and that mothers instinctively stroked their babies at CT optimal stroking speed and locations. Therefore, in the current study we aimed at examining two research questions. Firstly, whether there is a difference between the impact of stroking affective touch of mothers versus fathers on infants' RSA. Secondly, whether there is a difference in the stroking speed and body location that fathers and mothers chose to stroke their infant.

To examine these research questions we compared the impact of stroking touch on infant RSA from fathers with that from mothers in a pre-during-post design. We monitored infant ECG and respiration and made video recordings of the stroking activity to compare the stroking speed and body location chosen by the fathers versus mothers. In agreement with the ethical commission, we chose to not compare mother-father partners in order to avoid an atmosphere of competition within the early parent system. The participants in the father group were thus unrelated to the participants in the mother group. Also, we chose to respect the infant's ecological environment and collected the data in the respective mothers' or fathers' homes.

MATERIALS AND METHODS

Participants

The study was approved by Medical Ethics Committee of the University Hospital Of Brussels (B.U.N. 143201629352). We recruited 25 mothers and 25 fathers from prenatal classes and a private midwife's office. The average age of the mothers was 31.45 (*SD* = 3.74; range 25–41 years) and of their infants was 10.40 weeks (*SD* = 2.63; range 6–14 weeks). The average age of the fathers was 34.53 years (*SD* = 4.16; range 29–41 years) and of their infants was 10.86 weeks (*SD* = 3.53; range 6–14). All of the infants (25 males, 25 females) were healthy full-term born babies and had an Apgar score above seven (Apgar, 1966). They had a mean birth length of 49.718 cm (*SD* = 2.42; range 42–55 cm) and mean birth weight of 3.36 kg (*SD* = 0.50; range 2.5–4.7 kg). In the mother group, one infant was excluded because of fussiness (*N* = 24) and in the father group, five infants were excluded, two because of fussiness and three because the father changed the stroking location during the experiment (*N* = 20). Within this final population, there were 10 father-boy and 10 father-girl dyads and 13 mother-boy and 11 mother-girl dyads.

Apparatus

We made use of the BioRadio TM system (Great Lakes NeuroTechnologies Inc., Cleveland, OH, United States) to register the ECG and respiration. This system delivers synchronized registration of multiple ECG and respiration signals. The BioRadio Primary Module is a wireless acquisition system that has been used in previous infant studies (e.g., Van Puyvelde et al., 2014, 2015, 2019). The video recordings were made with a Sony Handycam type HDR-CX160. To perform the statistical analyses, we used the Statistical Package for Social Sciences Version 25.0 (SPSS 25.0). To analyze the physiological signals, we made use of VivoSense software version 3.1 (Vivonoetics, San Diego, CA, United States) and to calculate the stroking speed, we utilized the movement tracking software, Tracker 4.9.8, from the open source Physics by Brown (2018).

Monitoring of the Physiological Signals

We registered ECG by a standard single-channel ECG registrations (II derivation) as required by standard configurations with one electrode on the upper right side and the lower left side of the chest and one grounding electrode on the upper left side of the chest. The ECG signals were recorded with a 960-Hz sampling frequency filtered by a lowpass Bessel filter order 4 with a lower cutoff of 100 Hz. For the breathing we applied a lowpass Bessel filter order 2 with a lower cutoff at 1 Hz. The breathing movements of the mother/father were measured by a thoraco-abdominal respiratory effort belt, and those of the infant with a pediatric belt.

Questionnaires

We used the Touch Experiences and Attitudes Questionnaire (TEAQ) (Trotter et al., 2018) and the Paternal Postnatal Attitude Scale (PPAS) (Condon et al., 2008). The TEAQ inquires upon the attitudes and experiences with regard to affective touch. The TEAQ consists of six subscales, i.e., Current Social Touch (CST), Childhood Touch (ChT), Current Intimate Touch (CIT), Attitude to Intimate Touch (AIT), Attitude to Personal Grooming (APG), and the Attitude to Unfamiliar Touch (AUT). The PPAS examines the attitude of fathers toward their infant. It comprises the following components: Patience and Tolerance (PaT), Pleasure in Interaction (PiT) and Affection and Pride (AaP).

Procedure

The data were collected during home visits. In correspondence with Van Puyvelde et al. (2019), we asked the mothers/fathers to provide a room temperature of 22–24°Celsius (Blume-Peytavi et al., 2016). After the installation of the mobile lab, undressing the infant (except diaper) and fitting the electrodes and respiration belts, the mothers/fathers were asked to sit with their infant in a manner that felt comfortable to stroke. They were requested to not place the infant on their chest to exclude a confounding of cardiac transfer (as shown in Bloch-Salisbury et al., 2014; Van Puyvelde et al., 2015) independent from a potential stroking effect.

The experimental design contained three within-subjects' conditions, i.e., a pre-stroking baseline condition (PRE-STROKING, 3 min), a stroking period (STROKING, 3 min 20 s) and a post-stroking baseline condition (POST-STROKING, 3 min). During both PRE-STROKING and POST-STROKING, the mothers/fathers were asked to not interact with the infant. During the STROKING PERIOD, the mothers/fathers were asked to stroke their infant as they would normally do. We instructed them to stroke in a straight line but they were free to choose the body location. To avoid a potential risk on fatiguing CTs (Vallbo et al., 1999) we imposed an alternating scheme of 1 min stroking and 10 s pause, hence the STROKING duration of 3 min 20 s. The mother/father indicated when to start the experiment the PRE-STROKING condition. Afterward, the experimenter signed when to proceed to a next condition or when to stroke and when to pause. When the experiment was finished, we immediately removed the electrodes so that the parent could dress the infant again. Finally, the questionnaires were completed and a feedback was asked from the parent.

Physiological Signal Analysis

The ECG and respiration data were visually inspected for artifacts and (in)correct detections. When ectopic beats or erroneous detections were found, the data were manually corrected (removal of erroneous detection/artifact followed by a cubic spline interpolation; corrections <1%). Other undesired events in the physiology due to sneezing, coughing, caresses during baseline conditions, rocking the infant were removed as artifacts. These moments were selected by inspecting frame by frame the video recordings. In the parents' analysis, meanly 5.47 s per dyad was excluded which is <1% and in the infants mainly 5.93 s per dyad was excluded which is <1.1%. The timing of the detected R-wave was used to generate the RRI. For each testing block, infants' fR, RRI, and RSA were calculated. RSA was computed using the peak-valley method (Grossman et al., 1990), as advised for infant research (Ritz et al., 2012; Van Puyvelde et al., 2014, 2015, 2019). The VivoSense software provides programed algorithms that correspond with the advised standards of Grossman et al. (1990, 1991). For infant ECG, the ECG RR-lockout period for R-wave picking was adjusted to 0.1. For breathing detection, the algorithm sets a minimum peak-trough value that must be exceeded for a minimum-maximum pair to be denoted as an actual breath. This helps to eliminate non-respiratory small visceral movements from being marked as breaths. This minimum value is denoted as the minimum tidal volume in the properties of the breath channel. We adjusted the default value to infant norms of 5–30 ml. In line with Grossman et al. (1990), the inspiratory and expiratory windows were moved forward 750 ms in order to accommodate the phase shifts occurring between heart period and respiration (e.g., Eckberg, 2003). The VivoSense software also accounts for violations of the Nyquist criterion (i.e., the requirement that the sampling rate is at least twice as high as the frequency of interest) by scoring the breaths with no detectable peak-valley RSA as zero.

Data Preparation: Respiratory Controlled RSA (RSA*corr*)

Respiratory sinus arrhythmia is a multiple determined variable (Berntson et al., 2007) that reflects a combination of respiratory and somatomotor metabolic parameters (Beauchaine, 2001; Berntson et al., 2007). RSA is impacted by both respiration and speech (Tininenko et al., 2012). Therefore, it is necessary to disentangle effects of fR on RSA and to avoid speech during registration meant for RSA-measurement. Hence, we instructed the parents to not speak during the experiment. Further, to control for respiration effects, we applied the statistical within-subject regression approach (e.g., Grossman and Taylor, 2007) which is based on within-subject regressions on the averages of the data of each experimental block (see Grossman et al., 1991; Grossman and Taylor, 2007) with within-subjects z-transformations (Bush et al., 1993) as recommended in Van Puyvelde et al. (2014, 2015, 2019).

Video-Analysis

Stroking Speed

To analyze the mothers' and fathers' stroking speed, we made use of the video recordings. The stroking periods were cut into three blocks of one minute. First, a calibration was applied. We used auto-tracking analysis when a point mass (i.e., a central recognition point on the finger of the mother/father) was recognized through the entire length of the stroking period. If the point mass was not recognized through the entire stroking period, the manual tracking function was used (i.e., a frame by frame analysis). The stroking speed is the calculated distance divided by time. For analysis, we used the stroking speed

across the entire STROKING condition (Croy et al., 2016; Van Puyvelde et al., 2019).

Infant Motor Behavior

Based on the video recordings, we selected moments of motor activity and/or fussiness and removed them from analysis since this impacts RSA (Bazhenova et al., 2001; Ritz et al., 2012; Van Puyvelde et al., 2015, 2019). Based on the coding system of Bazhenova et al. (2001), we analyzed the motor behavioral states of the infants. Infants were in a quiet motor behavioral state for >95% of the time.

Statistical Analysis

All of the data were tested on normality (Kolmogorov–Smirnoff test), homogeneity and sphericity. Only in case of violation details will be reported.

Presentation of Respiratory Uncorrected Raw Physiological

The raw data were presented based on exploratory one-way repeated measures analyses of variance (ANOVA) for each group (infants stroked by mother and infants stroked by father) with condition (PRE-STROKING, STROKING, POST-STROKING) as within subjects variables and RSA, RRI, and *f*R as dependent variables.

Main Analysis

The main analysis consisted of three 3 × 2 (condition [PRE-STROKING, STROKING, POST-STROKING] × group [fathers, mothers]) mixed ANOVAs with condition as within-subjects, group as between subject factor and RSA*corr*, RRI and *f*R as dependent variable to evaluate the difference in impact of stroking touch on infants' RSA*corr*, RRI and *f*R delivered by mothers versus fathers.

Gender and Age Effects

Before testing for age and gender effects, we tested whether the infants in the mother versus father group were matched in age by an independent-samples *t*-test with age as dependent and group (mothers, fathers) as independent variable. Gender and age effects were tested by a series of 3 × 2 × 2 (condition [PRE-STROKING, STROKING, POST-STROKING] x group [mothers, fathers] x gender [boys, girls]) mixed ANOVAs and a 3 × 2 × 3 (condition [PRE-STROKING, STROKING, POST-STROKING] x group [mothers, fathers] x age [<8 weeks, 8–12 weeks, >12 weeks]) mixed ANOVA with condition as within-subjects factor, group and age/gender as between subject factor and RSA, RSA*corr,* RRI and *f*R as dependent variables.

Post hoc

For the repeated measure ANOVA tests, an extra evaluation of the proportion of variance (i.e., effect size or partial eta-squared, ηp^2) was made. We also performed a pairwise comparison by *post hoc* tests with the critical *p*-value for significance adjusted with Bonferroni correction. The confidence interval calculation was based on the within-subjects approach for repeated measures of Cousineau (2005). The method of Cousineau (2005) normalizes confidence intervals for within-subject designs.

Stroking Speed

A one-sample *t*-test compared the velocity rate of fathers and mothers with the mean velocity rate stroking speed observed in Croy et al. (2016).

Body Locations

The video recordings were also analyzed on the chosen body locations by both mothers and fathers.

Questionnaires

An independent-samples *t*-test tested the difference in scores self-reported by mothers and fathers on the TEAQ. A one-sample *t*-test compared the scores of the fathers on the PPAS in the current study with the mean scores reported in Condon et al. (2008).

RESULTS

Presentation of Respiratory Uncorrected Raw Physiological Data

Table 1 shows an overview of the raw data and one-way ANOVA (PRE-STROKING, STROKING, and POST-STROKING) tests on respiratory uncorrected RSA, RRI, and *f*R in the infants stroked by the mothers versus the infants stroked by the fathers. We inspected the data for outliers based on the *z*-standardizations ($z = \pm 2.58$). We excluded one infant in the group stroked by the mother having *z*-values > 2.56 ($N = 23$). The raw results show that there is an impact of stroking in both the mother and father group (see **Table 1**).

Main Analysis

There was a main effect of stroking touch on infants' RSA*corr,* $F(2,82) = 16.052$, $p < 0.001$, $\eta p^2 = 0.281$. RSA*corr* significantly increased from PRE-STROKING to STROKING ($p = 0.007$, Bonferroni corrected) and further to POST-STROKING ($p < 0.001$, Bonferroni corrected). There was no significant difference between the impact of stroking touch of mothers versus fathers (or no interaction effect condition-group) $F(2,82) = 0.342$, $p = 0.712$, $\eta p^2 = 0.008$. The elongated RSA*corr* main effect in POST-STROKING was mediated by both RRI and *f*R. That is, from STROKING to POST-STROKING, there was a significant main effect of stroking touch on infants' RRI, $F(2,84) = 7.831$, $p = 0.001$, $\eta p^2 = 0.157$, confirmed by Bonferroni ($p = 0.003$) with no difference between mothers and fathers (or no interaction effect condition-group), $F(2,84) = 0.487$, $p = 0.616$, $\eta p^2 = 0.011$ and a significant main effect on infants' *f*R, $F(2,84) = 9.751$, $p < 0.001$, $\eta p^2 = 0.188$, confirmed by Bonferroni ($p = 0.005$) with no difference between mothers and fathers (or no interaction effect condition-group), $F(2,84) = 0.522$, $p = 0.595$, $\eta p^2 = 0.012$ (see **Figure 1**).

Gender and Age Effects

There was no significant difference in age between the infants in the father group ($M = 10.52$ weeks; $SD = 2.67$) versus the infants in the mother group ($M = 9.99$ weeks; $SD = 2.28$), $t(42) = 0.714$, $p = 0.479$. We also found no age effects on RSA*corr,* RRI or

TABLE 1 | Means (SD) and analyses using one-way repeated measures ANOVA of raw RSA values (in ms), raw RRI values (in s), and raw fR values (respiration rate, cycles per minute) during PRE-TOUCH, TOUCH, and POST-TOUCH of the infants stroked by fathers versus infants stroked by mothers.

		PRE-TOUCH *M (SD)*	TOUCH *M (SD)*	POST-TOUCH *M (SD)*	*df*	*F*	*p*	ηp^2	Bonferroni
Infants in Father Group	RSA	7.900	8.986	10.132	2, 38	7.377	**0.002***	0.280	Post > Touch, $p = 0.016$
	raw	(1.668)	(1.081)	(1.674)					
	RRI	0.404	0.406	0.415	2, 38	2.493	0.096	0.116	/
		(0.014)	(0.013)	(0.016)					
	*f*R	53.73	53.05	49.87	2,	3.618	**0.036***	0.160	Post < Touch, $p = 0.036$
		(4.74)	(3.22)	(3.73)	38				
Infants in Mother Group	RSA	8.879	10.503	11.575	2, 44	12.598	**< 0.001***	0.364	Pre < Touch, $p = 0.027$
	raw	(1.311)	(1.728)	(1.422)					Pre < Post, $p < 0.001$
	RRI	0.427	0.423	0.439	2,	6.467	**0.003***	0.219	Touch < Post, $p = 0.003$
		(0.014)	(0.010)	(0.014)	46				
	*f*R	43.80	41.33	39.56	2,	7.418	**0.002***	0.244	Pre > Touch, $p, = 0.026$;
		(3.51)	(2.45)	(3.31)	46				Touch > Post, $p = 0.010$

Significant p-values are indicated in boldface with an asterisk (*).

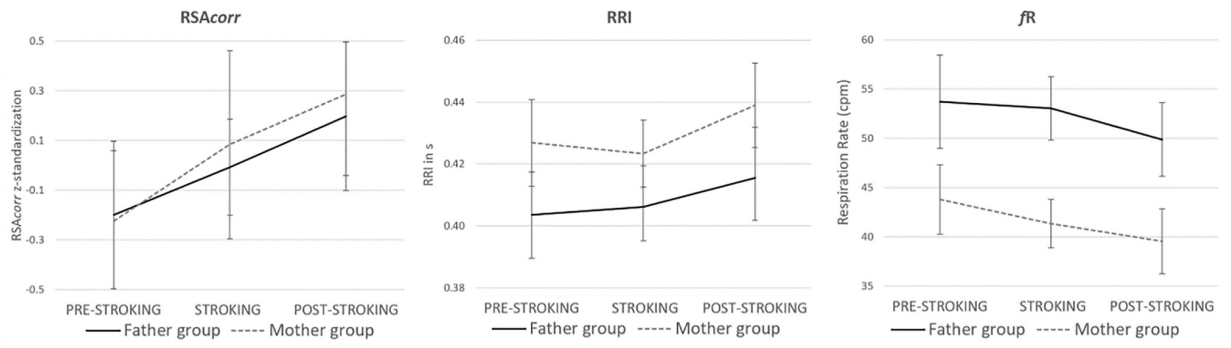

FIGURE 1 | Infants' RSA*corr*, RRI and *f*R before, during and after stroking touch in the Father group and Mother group. There was a significant main effect in RSA*corr*, RRI and *f*R and no difference between both groups. RSA significantly increased during and after stroking touch, RRI significantly increased after stroking touch and *f*R significantly decreased after stroking touch.

*f*R and one marginal gender effect on RRI, $F(2,84) = 2.918$, $p = 0.059$, $\eta p^2 = 0.061$ with a significant contrast from PRE-STROKING to POST-STROKING, $p = 0.32$ that we want to report. This contrast showed that infant boys in the father group had a larger increase in RRI from PRE-STROKING to POST-STROKING than the girls in the father group and the boys and girls in the mother group.

Stroking Speed

In the father recordings, the point mass was not always visible, hence video-analyses were based on 23 mother and 12 father recordings. There was no significant difference between the stroking speed of mothers ($M = 7.91$; $SD = 2.98$) and fathers ($M = 7.10$; $SD = 3.16$), $t(33) = 0.761$, $p = 0.452$. The stroking speed in the current study ($M = 7.38$ cm/s; $SD = 2.99$) was slightly slower than in Croy et al. (2016) ($M = 8.4$ cm/s), $t(34) = -2.028$, $p = 0.050$.

Body Location

Both mothers and fathers chose body locations that are known to contain CT afferents, that is head-region, body-region and the limbs (see **Table 2**).

Questionnaires

Normality was violated for one subscale of the PPAS, i.e., Affection and Pride, $D(24) = 0.23$, $p = 0.002$. Hence, no results will be interpreted with regard to this subscale. With regard to the TEAQ, fathers scored significantly lower than mothers on the items related to APG, $t(40.25) = -2.808$, $p = 0.010$. We found no correlations between the TEAQ-items and infant physiological variables. The fathers in the current study ($M = 73.4$; $SD = 10.2$) scored lower on the PPAS than in Condon et al. (2008) [$M = 79.2$; $SD = 9.0$), $t(18) = -2.467$, $p = 0.024$] due to a significant lower score on the items related to Patience and Tolerance, $t(18) = -3.378$, $p = 0.003$. We found no correlations between the PPAS-items and infant physiological variables.

TABLE 2 | Overview of the chosen body-locations by mothers and fathers to stroke their infant.

	Number of mothers	Number of fathers
Head-region	13	9
Body-region (back, shoulders)	5	5
Limbs	6	6

DISCUSSION

In the current study we compared the impact of paternal and maternal affective stroking touch on the RSA, RRI and *f*R of their infants. We replicated the experimental pre-during-post design of Van Puyvelde et al. (2019) with infant ECG and respiration monitoring and simultaneous video recording of the parents' stroking activity. In order to avoid an atmosphere of competition between parents we opted to study fathers and mothers who were unrelated to one another. Our current results showed no significant difference between the impact of paternal and maternal affective touch on our measures. When corrected for respiration, the infants' RSA*corr* significantly increased during and after they were touched, no matter whether the touch was delivered by fathers or mothers. This shows that parental affective touch has a beneficial impact on the parasympathetic regulation, be it delivered by mothers or fathers. Moreover, in line with the mother data in Croy et al. (2016) and Van Puyvelde et al. (2019), both the mothers and the fathers in the current study stroked their infants instinctively within a CT-afferent optimal velocity range of 1–10 cm/s with an average of 7–8 cm/s and chose body locations that are known to be rich of CT afferents such as the arms, legs, shoulders and head.

The first research question has been answered affirmatively: there was no difference between the impact of stroking touch delivered by mothers with whom infants already shared a prenatal physiological history versus fathers with whom the infants did not share a prenatal physiological history. Already in the 1970's, a series of studies showed that fathers interacted with a similar sensitivity toward their infants as mothers (e.g., Lynn and Sawrey, 1959; Hetherington, 1970; Biller, 1971, 1974; Block, 1973; Lynn, 1974; Maccoby and Jacklin, 1974; Sawin and Parke, 1979). Nevertheless, until recently (Bretherton, 2010; Brady et al., 2016), a persistent idea that giving comfort and care is the province of the mother remains. Bretherton (2010) pointed out that whatever the type of interaction parents chose to have with their infants, it is important to override doubts and to appreciate one another's actions. Therefore, the current study provides evidence that should obviate fathers' doubts upon the fact that they are able to evoke physiological regulatory processes in their infants during the 1st weeks of life.

The second research question has also been answered affirmatively (although with caution since only half of the father video recordings could be analyzed): there was no difference between the stroking velocity of mothers and fathers. Moreover, in line with earlier studies (Croy et al., 2016; Van Puyvelde et al., 2019), the stroking speed of both the analyzed fathers and mothers was within the optimal CT afferent stimulation window and the chosen locations of both fathers and mothers were CT afferent rich body locations (i.e., shoulders, arms, legs, head). These results give scope to discussion with regard to underlying mechanisms. The observations with regard to the stroking speed in both mothers and fathers may suggest that it was the stimulation of CT afferents that underpinned the infants' parasympatho-inhibitory regulation, as was suggested in Van Puyvelde et al. (2019). Another clarification could be found in a simple time-effect, that is that RSA would have increased as a result of the time being together with the father. However, in mothers, a time-effect was excluded by Van Puyvelde et al. (2019) since non-stroked infants' RSA decreased after the termination of static touch. Hence, we doubt that similar maternal and paternal touch patterns with similar impacts on infants' RSA would result from two different underlying mechanisms. However, it is plausible that infants need some familiar social cues to identify themselves with affective touch as stated by Pirazzoli et al. (2019) and those cues were present in both the fathers and the mothers arms. To further examine the role of underlying mechanisms and influencing factors with regard to touch-related parasympathetic infant regulation, two aspects are on the research agenda. Firstly, different types of touch-providers should participate, that is strangers as well as family members in atypical family constellations such as same-gender parents and adoption parents. There is a lack of research that determines the aspects that are important to build the needed physiological and affective conditioning processes in a context different to the common biological mother-infant interaction. The current study showed that a shared prenatal context is not a critical condition to help an infant in the stimulation of parasympatho-inhibitory regulation. Hence, this study may open doors toward new research that examines parasympatho-inhibitory transfer processes within other parent-infant constellations. Secondly, to pinpoint the mechanism of CT afferents, standardized velocity rates should be compared as in Fairhurst et al. (2014) (however in longer windows in order to allow RSA-analysis on top of an acute phasic response in HR) as well as the effect of the temperature of the delivered touch—bearing in mind CTs are velocity tuned and, interestingly, their firing rate varies depending on the temperature of a touch (see Ackerley et al., 2018). Finally, all of these avenues of research should be investigated in an ecological as well as non-ecological context. The comparison with a non-ecological context could increase insight in what manner the conditioned aspect of the ecological nurturing context is important in an eventual developmental sensitization process of CT afferents. For instance, in a study by Pirazzoli et al. (2019), a brain response to affective touch in 5-months old infants could not be found, which the authors ascribed to the strict laboratory settings that were applied in place of a more natural mother-infant context.

Parasympathetic regulation was measured by registering both ECG and respiration of the infants. This remains the only way to have full insight into the cardiorespiratory system. Besides parasympathetic activity, respiratory and somato-motor metabolic parameters (Beauchaine, 2001) need to be taken into account when it comes to final interpretation. However, the real interconnectedness between heart rate (HR) and respiration, which is in fact the base of RSA, remains unknown when analyses are based on HR only (Grossman et al., 1990). Our analyses showed that the responses to parental touch in the infants were mediated by both their HR and respiration. There was a combined significant increase in RRI and decrease in *f*R. During POST-STROKING, in both

the father and mother group, the infants' heart period and respiration were engaged in what can be called a reciprocally parasympathetic activation (Berntson et al., 1991). An autonomic reactivity response often yields a combined respiratory induced sympathetic deactivation with a cardio induced parasympathetic activation (Berntson et al., 1991). The delayed reciprocally parasympathetic activation during POST-STROKING in the current study may point to the fact that affective touch contributes not only to an acute phasic reactivity response but also to the installation of a more integrated tonic response of long-term regulation. Further research needs to establish whether this delayed slower reactivity response might be the expression of the mechanism that underpins the earlier suggested role of touch within the development of an optimally functioning stress regulation system (e.g., Levine, 2001; Meaney, 2001; Champagne et al., 2008; Uchida et al., 2010; Franklin et al., 2011; Walker and McGlone, 2013; Curley and Champagne, 2016; Van Puyvelde et al., 2019).

The inclusion of respiration measures also revealed an interesting difference between the infants in the mother versus father group. In the mother group, a respiratory induced effect occurred already during the STROKING period whereas in the father group this effect was delayed and only observed during POST-STROKING. In the mother group, during the STROKING period, increased RSA occurred in combination with decreased fR whereas the decreased fR in response to touch delivered by fathers only occurred in the POST-STROKING period. Moreover, the infants in the father group had throughout the three conditions a significantly higher fR and RRI than those in the mother group. These differences were not due to a difference in age since the infants were matched in age. Moreover, no age-effects were found. Previous studies reported that mothers interact in a more nurturing and fathers in a more playful and stimulating manner with their infants (e.g., Rendina and Dickerscheid, 1976; Lamb, 1977, 1981; Feldman, 2003; Kokkinaki and Vasdekis, 2014). From a physiological perspective, it has been suggested that with these different interactive arousal patterns, mothers offer their infant the practice of affective regulatory coordination (Feldman, 2003) whereas fathers would offer them a manner to cope with high-intensity activity in a positive arousal state, increasing the infant's stress resilience (Pedersen et al., 1979). In the current study we did not collect information on the amount and type of interaction the fathers and mothers respectively maintained and whether a similar distinction in interactive behavior might have caused different anticipatory physiological states in the infant. Therefore, to find out whether the difference between infants' fR and RRI in the mother group versus father group was due to a coincidental interindividual variance or not, a baseline study should be conducted comparing a baseline measured independent from the respective parent with a baseline measured in the arms of the father and the mother.

Finally, we found a tendency for a beneficial impact of a father-son gender-match with regard to RRI from STROKING to POST-STROKING. Although only the contrast was significant, we want to mention this result since it corresponds with earlier behavioral observation research that reported higher levels of matched synchrony (Feldman, 2003), emotional sharing and physical proximity (Lamb, 1977, 1981; Weinraub and Frankel, 1977) in gender-matched parent-infant interactions than in non-gender-matched interactions which had been ascribed in former research to a shared biological preparedness between matched genders (Feldman, 2003).

This research had some strengths and limitations. This is one of the few studies that investigated the impact of paternal behavior on infants' physiology and that respected the infant's ecological home-situation. It offered a video-controlled analysis and included respiration to interpret RSA and correct it for respiratory confounds. A limitation of this time-consuming approach is a rather low number of participants. Further, a non-stroking father group, a stranger stroking and stranger non-stroking group would had been beneficial to further increase insight in the underlying mechanisms such as CT-afferent activity and the interaction with the ecological context of the infant. Finally, information on the parent-infant interactions at home would had informed on gender-typical interaction patterns in the participants.

In summary, the current study found that there is no difference between the final impact of stroking touch delivered by mothers versus fathers on the RSA*corr* of infants. Parental touch, be it delivered by a mother or a father induces parasympatho-inhibitory regulation in the infant. This regulation is mediated by both HR and respiration. However, when delivered by fathers, the respiratory effect occurred in a later stage than in mothers, i.e., in the father group after touch delivery in place of during touch. Affective touch is a gender-neutral facilitator to create a 'parental nest' of parasympatho-inhibitory regulation. By getting in touch through bonding, both father and mother pave the way to keep in touch through attachment.

DATA AVAILABILITY

The datasets generated for this study will not be made publicly available. Currently this is not standardly included in our ethical procedures.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ICH-GCP. The protocol was approved by the Medical Ethics Committee of the UZ Brussel/VUB. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Our data will not be made available in accordance with the ethical procedures of ICH-GCP and GDPR.

AUTHOR CONTRIBUTIONS

MVP: supervision, analysis, and writing. LC: data collection and analysis. A-SF: analysis. NP: reflecting team. FM: supervision and manuscript revision.

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Heart Rate Variability During a Joint Attention Task in Toddlers With Autism Spectrum Disorders

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Background: Autism Spectrum Disorders (ASD) are a heterogeneous group of neurodevelopmental disorders featuring early impairments in social domain, with autonomic nervous system (ANS) unbalance possibly representing a useful marker for such disturbances. Impairments in joint attention (JA) are one of the earliest markers of social deficits in ASD. In this study, we assessed the feasibility of using wearable technologies for characterizing the ANS response in ASD toddlers during the presentation of JA stimuli.

Methods: Twenty ASD toddlers and 20 age- and gender-matched typically developed (TD) children were recorded at baseline and during a JA task through an unobtrusive chest strap for electrocardiography (ECG). Specific algorithms for feature extraction, including Heart Rate (HR), Standard Deviation of the Normal-to-Normal Intervals (SDNN), Coefficient of Variation (CV), pNN10 as well as low frequency (LF) and high frequency (HF), were applied to the ECG signal and a statistical comparison between the two groups was performed.

Results: As regards the single phases, SDNN ($p = 0.04$) and CV ($p = 0.021$) were increased in ASD at baseline together with increased LF absolute power ($p = 0.034$). Moreover, CV remained higher in ASD during the task ($p = 0.03$). Considering the phase and group interaction, LF increased from baseline to task in TD group ($p = 0.04$) while it decreased in the ASD group ($p = 0.04$).

Conclusions: The results of this study indicate the feasibility of characterizing the ANS response in ASD toddlers through a minimally obtrusive tool. Our analysis showed an increased SDNN and CV in toddlers with ASD particularly at baseline compared to TD and lower LF during the task. These findings could suggest the possibility of using the proposed approach for evaluating physiological correlates of JA response in young children with ASD.

Keywords: autism spectrum disorders, autonomic nervous system, joint attention, wearable sensors, eye-tracking

INTRODUCTION

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental conditions featuring impairments in social communication, as well as restricted or stereotyped interests and behaviors (American Psychiatric Association, 2013). Its prevalence is constantly increasing-up to around 1 out of 50 children (Blumberg et al., 2013)-but the cause of such condition is yet unknown, despite being suggested that a gene-environment interaction probably could be at the basis of the disorder (Meek et al., 2013).

Due to the wide clinical and etiological heterogeneity of ASD, the early identification of a set of informative risk markers represents a key-challenge to enable early detection and early diagnosis of ASD (Sacrey et al., 2015), crucial steps for a clinical assessment of this disorder. In fact, early diagnosis allows creating the field for undertaking early specific intervention, in turn bringing in benefits for both the children, even as young as 18-month-old (Rogers et al., 2014; Brian et al., 2015), and the community at large (Schreibman et al., 2015).

Several studies suggest that Autonomic Nervous System (ANS) activity is linked to social functioning in individuals with ASD, suggesting a role of this system in regulating social interactions in this condition (Dawson and Lewy, 1989; Romanczyk and Gillis, 2006; Faja et al., 2013; Sheinkopf et al., 2013; Neuhaus et al., 2014). Social functioning and communication are managed by both sympathetic (SNS) and parasympathetic (PNS) branches of the ANS (Porges, 2001, 2003). The PNS appears to have an important role in social functioning, being partially mediated by the myelinated vagus, in turn innervating several organs in the face and in the neck, and affecting various functions, including cardiac activity, influencing social behavior and communication (Neuhaus et al., 2016). In socially rich settings, an increased PNS activity eases approach to others, as well as adaptive social behavior (Porges, 2001). In addition, social engagement takes advantage of SNS activation, as well. SNS brings to increased heart rate (HR), sweating and alert state, mainly through acting on the so-called "fight or flight" mechanism (Stifter et al., 2011; Diamond and Cribbet, 2013). Summarizing, both SNS and PNS are somewhat involved in social behavior, with SNS activation reflecting a threat-oriented response, while PNS dominance easing adaptive social engagement (Bal et al., 2010).

Several approaches could be employed to assess SNS and PNS, including minimally obtrusive methods based on electrodermal activity (GSR, Galvanic Skin Response) and electrocardiography (ECG). Concerning GSR, it is thought that a reduced electrodermal activity is associated with decreased SNS influence (Hubert et al., 2009), whereas an increase in skin conductance during specific social tasks subtends higher SNS activation (Mathersul et al., 2013). Concerning the ECG signal, the PNS contribution (and, consequently, the corresponding SNS activation level) can be unobtrusively assessed by studying the heart rate variability (HRV) (Porges, 2001) and, more specifically, the contribution to beat-to-beat variability at higher frequency, as well as the respiratory sinus arrhythmia (RSA) (Berntson et al., 1997). RSA, in particular, was seen to predict social responsiveness early during childhood (Patriquin et al., 2014), with higher PNS detected by RSA analysis associated with an increase in social competence and engagement, adaptive coping strategies, and spontaneous eye gazes (Fabes et al., 1993; Eisenberg et al., 1995; Henderson et al., 2004; Heilman et al., 2007). Several works have been conducted in autistic children, highlighting a lower baseline RSA with respect to typically developing (TD) peers (Guy et al., 2014), associated with weaker social and communication skills (Watson et al., 2010; Neuhaus et al., 2016), and significantly altered reactions to social-like stimuli (Van Hecke et al., 2009), which suggest a role of RSA in social reactivity. However, the merging of markers of sympathetic and parasympathetic activation has been rarely assessed in young children with ASD (Neuhaus et al., 2016).

Joint attention (JA), which is defined as the ability to coordinate visual attention with another person and then shift the gaze toward an object or event (Mundy and Gomes, 1998), is one of the more precocious and consistent early signs of ASD (Charman, 2003). Impairment in JA is primarily a social or social-cognitive phenomenon as it implies to look into the eyes of a social partner and to coordinate their visual attention with him (responding JA). Empirical studies suggest that JA in infancy is pivotal for learning, language development, and social-cognitive development in the first years of life (Rothbart and Bates, 1998; Thurm et al., 2007; Mundy and Jarrold, 2010), and is a significant predictor of social competence and cognitive outcomes in TD children as well as those with neurodevelopmental disorders. Thus, measuring behavioral or physiological parameters during a JA task is a marker of social processes in ASD. Several studies have described abnormal social response in subjects with ASD during live JA tasks, as well as eye-tracking (Chawarska et al., 2003; Bedford et al., 2012; Falck-Ytter et al., 2012, 2015; Billeci et al., 2016a) or neuroimaging experimental paradigm (Redcay et al., 2012; Jaime et al., 2016).

Wearable systems and wireless technologies, allowing monitoring patients in an unobtrusive way, are particularly suitable for the recording of physiological parameters in very young children with neuropsychiatric conditions such as ASD during social tasks. Few previous investigations measured HRV in very young children with ASD, with some (Zantinge et al., 2017a,b) did not use wearable devices, possibly increasing discomfort of ASD patients, motion artifacts in the heart rate processing, and consequently to exclusion of some non-analyzable data. To our knowledge only one study (Watson et al., 2012 used wireless ECG sensors, in very young children with ASD, however involving slightly older children than in the present study. Specifically, the authors used the Mini-Logger unit, which was placed in the child's pocket or a waist pack and attached to two surface electrodes placed on the child's chest. Twenty-two children with ASD (26.1 $\pm$ 3.3 months), 15 typically developing boys 15 (33.0 $\pm$ 6.1 months) 14 typically developing boys (12.9 $\pm$ 3.8 months), recruited as a language age comparison group, were assessed with behavioral (looking) and physiological (heart rate and respiratory sinus arrhythmia) measures while looking to nonsocial and child-directed speech stimuli. Although some children were excluded to technical problems or excessive movement, most of the children completed the tasks without

complications. The authors found an increased heart rate in children with ASD suggesting that young children with ASD have an elevated arousal level.

Given that literature in this field is still poor, the aims of this work were to test the feasibility of using a wearable chest belt for the monitoring of ECG signal in toddlers with ASD, and to measure the ANS response in a group of toddlers with ASD and neurotypical age- and gender-matched controls during a JA eye-tracking task.

MATERIALS AND METHODS

Study Population

In the present study, we initially enrolled 46 subjects, equally divided into two groups (ASD and TD children). The ASD group was recruited in three different Institutions: the Autism Unit of IRCCS Stella Maris Foundation of Pisa, the Division of Child Neuropsychiatry of the University Hospital of Messina and the Hospital of Matera. The clinical diagnosis of ASD was established according to the Diagnostic and Statistical Manual of mental disorders-5 criteria (American Psychiatric Association, 2013) and confirmed using algorithm cutoffs on the Autism Diagnostic Observation Schedule-Generic (ADOS-G) (Lord et al., 2012), which was administered by ADOS research reliable examiners. In addition, the parents of the children with ASD completed the Modified Checklist for Autism Toddlers (M-CHAT) (Robins et al., 2001). The exclusion criteria were as follows: (a) neurological syndromes or focal neurological signs; (b) significant sensory impairment; (c) anamnesis of birth asphyxia, premature birth, head injury or epilepsy; (d) use of any psychotropic medication; and (e) potential secondary causes of ASD determined by high-resolution karyotyping, DNA analysis of Fragile-X or screening tests for inborn errors of metabolism.

The participants with TD were recruited from daycares in the Pisa, Messina and Matera metropolitan areas. All children (ASD and TD) received a nonverbal developmental evaluation through the administration of the performance subscale of the Griffiths Mental Developmental Scales (Griffiths, 1984). **Figure 1** shows the distribution of the Griffiths Total Scale for ASD and TD.

The inclusion criteria for TD children were an age between 18 and 36 months and the Child Behavior Checklist 1½−5 (CBCL; Achenbach and Rescorla, 2000) Total score under the borderline/clinical range. Exclusion criteria were family risk for ASD or other neurodevelopmental disorders, visual or auditory impairments and any concerns from the caregivers. After the application of the inclusion and exclusion criteria, the final sample was composed of 40 subjects, equally divided into the two groups (see Results for details). A description of the final study population is displayed in **Table 1**.

An informed consent was obtained from all parents of the children enrolled, after receiving an exhaustive explanation of the study. The experimental procedures and the informed consent were approved by the ethics committee of the IRCCS Stella Maris Foundation (Calambrone, Pisa, Italy). The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Assessment Scales

The ADOS-G (Lord et al., 2012), administered by AN, certified to administer ADOS in clinical and research setting, is an observation measure of current autism symptom severity; on the basis of language level, the module 1 was used for all children. We have also used the Calibrated Severity Scores (CSS), a standardized metric developed to assess autism symptoms as a clinical entity distinct from cognitive and adaptive differences. This metric provides a means to assess symptoms of autism over time in a range between 1 and 10, where 1–3 accounts for no spectrum, 4–5 for ASD, and 6–10 for autism. In the original validation study, these scores were shown to be less influenced by verbal IQ, which accounted for 43% of variance in raw ADOS scores and only 10% in CSS scores (Gotham et al., 2009).

The M-CHAT (Robins et al., 2001) is validated for screening toddlers between 16 and 30 months of age, to assess risk for ASD. The M-CHAT can be administered and scored as part of a well-child check-up, and can be also used by specialists or other professionals to assess the risk for ASD. The primary goal of the M-CHAT was to maximize sensitivity, meaning to detect as many cases of ASD as possible.

The Griffiths Mental Developmental Scales (Griffiths, 1984), administered by expert clinicians, are a standardized developmental test for children from birth to 96 months of age. They comprise six scales, but because of the young age of the children, only five out of the six were administered: Locomotor, Personal-Social, Language, Eye and Hand Coordination, and Performance. Raw scores have been computed for each subscale and converted to general quotient scores, using tables of the analysis manual.

The CBCL (Achenbach and Rescorla, 2000) is a 100 item parent-report measure designed to record the behavioral peculiarities of preschoolers. Each item describes a specific behavior, and the parent is asked to rate its frequency on a three-point Likert scale. The scoring gives, among others, three main scores (Internalizing, Externalizing, and Total Problems). A T-score (for Internalizing and Externalizing, and for Total Problems) of 63 and above is considered clinically significant, and values between 60 and 63 identify a borderline clinical range; values beneath 60 are considered not clinical.

Acquisition Protocol

Electrocardiography (ECG) signals were recorded during an eye-tracking JA task previously described by our group (Billeci et al., 2016a). Toddlers' gaze was recorded by means of the SMI 500 Eye Tracking device provided by SensoMotoric Instruments (Teltow, Germany), while video stimuli were presented on a 22-inch flat monitor. The toddlers sat on a chair seat to limit movements. Simultaneously, ECG signals were wirelessly acquired.

Attention to the stimuli was assured by the simultaneous acquisition of eye-tracking and ECG data. Trials for which the children did not look at the screen were excluded from the analysis and children with more than 50% of excluded trials were eliminated from the final sample. Moreover, all the sessions were recorded with a webcam connected to the stimulus PC.

Briefly, the task consisted in watching some short videos reproducing three JA conditions: a response to JA (RJA) and

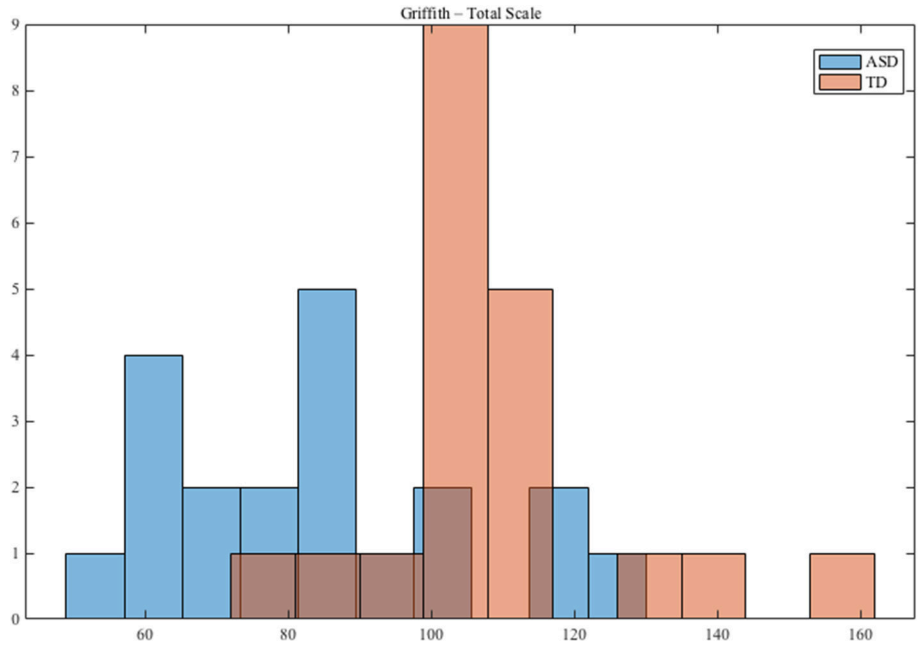

FIGURE 1 | Distribution of Griffith Performance for ASD and TD groups.

TABLE 1 | Study population (significance: $p < 0.05$).

	ASD	TD	*p*-value
N	20	20	1.00
Age (months, mean ± SD)	26.1 ± 3.3	26.2 ± 3.7	0.48
Gender (M/F)	14/6	15/5	0.80
Griffith Performance (mean ± SD)	81.9 ± 21.8	108.9 ± 19.6	0.001
ADOS-G, module 1–*Total*	14.9 ± 4.5	–	–
ADOS-G, module 1–*Communication*	4.9 ± 2	–	–
ADOS-G, module 1–*Interaction*	9.8 ± 3.4	–	–
ADOS-G, module 1–*Calibrated Severity Score*	7.3 ± 2.1	–	–

two initiation of JA (IJA) conditions. The RJA condition consisted of a woman placed between two identical objects, in turn placed in front and on either side of her; she smiled and turned her head toward one of the two objects. On the other hand, in the two IJA tasks, the woman maintained direct gaze but in one case one of the objects activated unexpectedly, while in the other one the object appeared from one end of the frame and crossed the scene. The video sequence lasted about 8 s and was repeated several times, so that the total duration of the task was about 5 min (Billeci et al., 2016a). A total of 12 trials was presented to each child.

The acquisition protocol also included a "baseline" phase of 5 min before the beginning of the task, in which the child was sitting on a chair near to the therapist, without any particular annoyance due to the clinical scenario.

ECG Monitoring

Electrocardiography (ECG) signals were recorded with a smart sensor of the CE certified Shimmer® platform (Burns et al., 2010), composed of two separate boards, one (base-board) that is similar to all the sensors of this factory, and that is programmed by using specifically designed codes, and the second (front-end board) that is able to perform a pre-filtering of the signal acquired reducing the artifacts of the signal. Overall, the ECG module is extremely small (50 × 25 × 23 mm) and light-weight (30 g).

The ECG sensor was modified by adding two pins in order to allow its interfacing with the common Polar™ cardio-fitness chest strap, featuring two dry electrodes in the inner side of the strap, interfaced to the skin surface of the subject for a single-lead acquisition. The chest strap employed is extremely customizable, allowing for monitoring physiological signals in children with different anatomical characteristics, such as the chest diameter. The signals were acquired with a sampling rate of 200 Hz and an A/D resolution of 12 bits. Home-made Matlab™ scripts were then used for ECG pre-processing and feature extraction.

ECG Pre-processing

A stepwise filtering was initially applied to remove artifacts and interferences. Body movements and respiration were removed with a cubic spline 3rd order interpolation between the fiducial isoelectric points of the ECG. A notch filter was also applied to remove the power line interference at 50 Hz and an IIR low pass filter at 40 Hz was applied to eliminate muscular noise. In addition, an interpolation using the Fourier method was applied to the signal to improve RR fiducial points recognition (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). The

Pan-Tompkins method was then adopted to detect the QRS complexes (Pan and Tompkins, 1985), from which the RR series was extracted. Residual artifacts and outliers were removed from RR series by visual inspection. Outliers were replaced by division or summation. The division was applied when the outlier was determined by a failure to detect an R-peak while summation was applied when it was caused by faulty detections of two or more peaks within a period representing the RR interval (RRI).

Feature Extraction

RR series were further analyzed using home-made Matlab™ scripts to extract several time- and frequency-domain features both from the baseline and the task phases.

Time-Domain Analysis

The following time-domain features were extracted and used for analysis:

- Heart rate (HR), expressed as beats per minute (bpm).
- Standard deviation of NN intervals (SDNN), which is a measure of both sympathetic and parasympathetic activity and therefore provides an index of total HRV (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).
- The coefficient of variation (CV) of the time interval between two consecutive R-waves (RRI) calculated by dividing the standard deviation of RRI (SDNN) by the mean of RRI. We corrected the SDNN with respect to the mean of RRI as HRV and HR are mathematically associated (Sacha and Pluta, 2008; Sacha, 2013).
- pNN10 defined as the percentage of successive normal IBI $> \times > 10$ ms and assessing parasympathetic activity (Mietus et al., 2002).

Frequency-Domain Analysis

The power spectral density was estimated by the Welch method (Welch, 1967), through which we extracted the following frequency-domain features (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996):

- Low Frequency (LF) which is the absolute value of the Low Frequency (LF) power (0.04–0.24 Hz) and emphasizes changes in sympathetic regulation.
- High Frequency (HF) which is the absolute value of the High Frequency (HF) power (0.24–1.04 Hz). This frequency band corresponds to band of the spontaneous breathing frequency of children (i.e., from 0.24 to 1.04 Hz or approximately 15–60 breaths per minute) (Bar-Haim et al., 2000; Heilman et al., 2007; Patriquin et al., 2014). Thus, this band in children overlaps with Respiratory Sinus Arrhythmia (RSA). This measure is mostly related to parasympathetic regulation.
- Normalized Low Frequency (nLF), which is the ratio between LF and the sum of LF and HF.
- Normalized High Frequency (nHF), defined as the ratio between HF and the sum of LF and HF.
- LF/HF Ratio, representing the ratio between the power of LF and HF bands. Its measure indicates the overall balance between sympathetic and parasympathetic systems.

The frequency band limits of LF and HF were selected according to the recommendations for reporting HRV in children and infants (Quintana et al., 2016).

Statistical Analysis

Statistics was performed using SPSS 23 software (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was applied to evaluate whether the variables considered were normally distributed. A repeated-measures ANCOVA was performed with "phase" (i.e., baseline or JA task) as a "within-group" and "group" (i.e., ASD or TD) as a "between groups" factor. When the variables had a non-normal distribution, variables and covariate were transformed in ranks and the analysis of covariance on ranks was performed. Given that the two groups were different in terms of Griffiths performance, this measure was used as covariate in the ANCOVA. In case of a significant phase or phase x group effect ($p < 0.05$), a *post-hoc* analysis was performed to compare differences between phases within each of the two groups (paired-sample *t*-test or Wilcoxon test, according to the distribution of variables) and differences between the two groups (*t*-test or Mann-Whitney test, according to the distribution of variables). Given the exploratory nature of this study, we also reported differences with significance between the two groups within the different phases for all the measures as resulted from independent samples tests (*t*-test or Mann Whitney test, according to the distribution of variables). Statistical correlations between autonomic parameters and selected items from ADOS-G (pointing; response to JA, gesturing, showing, initiation joint attention and unusual eye contact) and Griffith Scales were performed using the Pearson or Spearman Correlation Test.

RESULTS

Participants Characteristics

After the exclusion criteria application, 3 TD were excluded because the CBCL Total score was over the threshold. Additional 3 ASD children were excluded, one of which due to premature birth, and the other two due to the use of psychotropic drugs.

Thus, the final sample included into the statistical analysis, was constituted of 40 children, 20 TD and 20 ASD.

Feasibility Assessment

The first analysis aimed at assessing feasibility of the approach proposed in monitoring toddlers with ASD and TD controls. The tolerability and comfort of toddlers were judged by a psychologist who was present during the sessions and was further confirmed by the inspection of the videos recorded by the webcam. These observations showed that the system did not cause any kind of annoyance and all 40 children successfully accomplished the experimental protocol proposed without showing sensory-motor and/or behavioral issues in wearing the devices and without any difficulties or constraints. Excessive movements were limited also because in the protocol the toddlers were seated in a chair while watching the stimuli and thus relatively restrained in their

physical activity. Both the ASD and the TD group attended the videos at the same way for an acceptable time. Indeed, repeated-measures analysis of variance revealed that there was no significant effect of task or group × task on the number of usable trials (Billeci et al., 2016a). The mean number of usable trials was 11.7 ± 1.2 for the ASD group and 11.2 ± 1.5 for the TD group.

ECG Signal Analysis

The second analysis aimed at comparing the two study cohorts according to the features extracted from the ECG signal.

According to the Shapiro-Wilk test, LF and HF (both during baseline and task) had a non-normal distribution while the other variables were distributed normally. Thus, we performed parametric or non-parametric tests according to the variables distribution.

There was a significant effect of phase x group for LF ($F = 5.54$, $p = 0.026$) (**Figure 2**). In TD group, LF increased from baseline to task (median ± IQR: 316.00 ± 1162.00 vs. 537.00 ± 3339.50, $p = 0.04$) while in the ASD group it decreased (median ± IQR: 774.00 ± 6601.00 vs. 357.00 ± 1081.60, $p = 0.04$). There was not any significant phase or phase × group interaction for the other time and frequency measures.

Comparing the two groups, we observed that SDNN, CV, and LF were significantly higher in the ASD group compared to the TD group at baseline. In addition, CV was also higher in in the ASD group during the task. All the other time- and frequency-domain features did not differ between-groups, nor within-group. In **Table 2** we reported values of all the features for the two groups and the significance obtained independent samples tests.

Correlation Between RR Features and Clinical Measurements

Concerning the correlation analysis, in the ASD group, CV at baseline was positively correlated with the item "initiation joint attention" of the ADOS-G ($r = 0.506$, $p = 0.032$).

In the TD group, a significant negative correlation between LF at task and Internalizing item of the CBCL Scale ($r = -0.493$, $p = 0.046$) was observed.

DISCUSSION

The first aim of our study was to test the feasibility of using wireless and wearable technology in toddlers with ASD and TD to record ECG signals, since these technologies are scarcely applied in these populations. The results of our study provided evidence of the feasibility of using a wearable unobtrusive chest strap for assessing ANS activation through ECG signal recording and analysis in such a sample. This could be particularly important in young age as it could provide important physiological markers of social functioning in toddlers with ASD, thus contributing to the diagnosis and the identification of the best therapeutic approach. The system proposed was already successfully applied by our group in schoolers with ASD (Billeci et al., 2016b; Di Palma et al., 2017), thus this study extends our previous results also to toddlers, therefore possibly representing the basis for future related investigations. Moreover, we showed the feasibility of simultaneous recording of eye-tracking and ECG during a social task, like JA, providing the possibility of measuring at the same time gaze pattern and physiological response. These two facts, taken together, foster the employability of this solution for

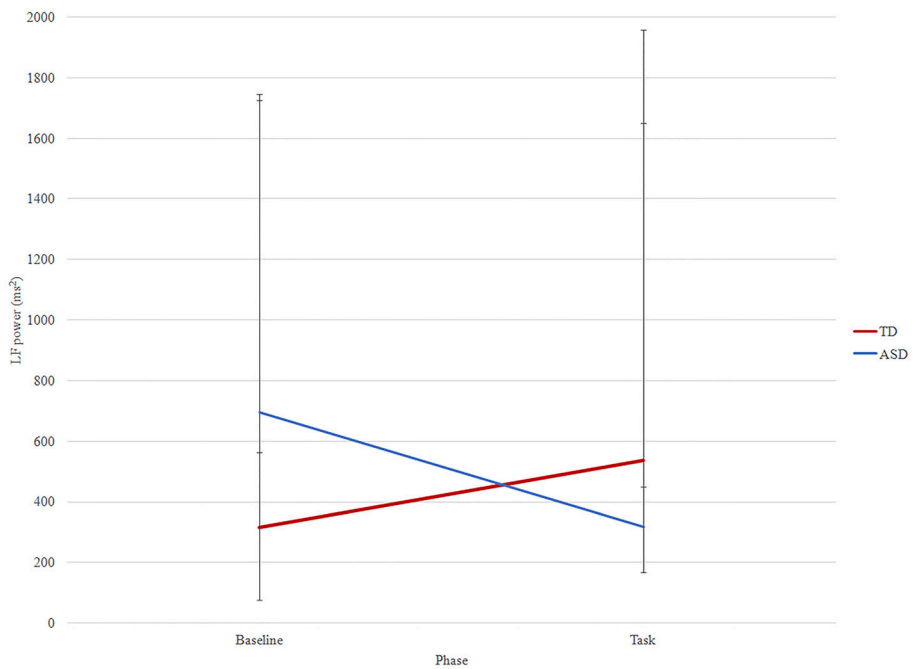

FIGURE 2 | LF changes between phases (baseline and task) in ASD (blue) and TD (red) groups. Median and interquartile are reported.

TABLE 2 | Comparison between the *two* study groups in all the single ECG features extracted in the single testing phases (significance: $p < 0.05$).

	ASD	TD	*p*-value
BASELINE			
HR (bpm, mean ± SD)	104.24 ± 36.41	112.49 ± 22.82	0.41
SDNN (ms, mean ± SD)	0.45 ± 0.63	0.14 ± 0.16	0.04
CV (n.u., mean ± SD)	0.78 ± 0.80	0.28 ± 0.32	0.021
pNN10 (%, mean ± SD)	74.21 ± 23.53	68.57 ± 24.57	0.49
LFn (n.u., mean ± SD)	0.43 ± 0.19	0.38 ± 0.16	0.59
HFn (n.u., mean ± SD)	0.57 ± 0.10	0.62 ± 0.08	0.95
LF/HF Ratio (mean ± SD)	3.81 ± 1.91	4.67 ± 2.04	0.26
LF [ms^2, median (IQR)]	696.00 (1669.00)	316.00 (1162.00)	0.047
HF [ms^2, median (IQR)]	1550.00 (3746.00)	344.50 (2587.00)	0.34
TASK			
HR (bpm, mean ± SD)	119.06 ± 43.53	120.56 ± 25.43	0.90
SDNN (ms, mean ± SD)	0.26 ± 0.32	0.15 ± 0.22	0.03
CV (n.u., mean ± SD)	0.53 ± 0.44	0.30 ± 0.35	0.09
pNNx (%, mean ± SD)	75.24 ± 19.55	68.01 ± 19.66	0.28
LFn (n.u., mean ± SD)	0.32 ± 0.21	0.41 ± 0.19	0.24
HFn (n.u., mean ± SD)	0.68 ± 0.44	0.59 ± 0.19	0.24
LF/HF Ratio (mean ± SD)	2.86 ± 2.12	3.33 ± 1.91	0.50
LF [ms^2, median (IQR)]	318.00 (1669.0)	537.00 (1199.30)	0.50
HF [ms^2, median (IQR)]	1300.00 (2738.00)	472.50 (3110.50)	0.91

For variables with normal distribution mean and standard deviation (SD) is reported, while for variables with non-normal distributions median and interquartile range (IQR) is reported. n.u.: normalized units.

monitoring HR/HRV also in naturalistic, less structured settings, and with particularly fragile populations, including toddlers with early evidence of neurodevelopmental disorders, strengthening the importance of the present solution.

The second aim of the study was to evaluate specific differences in ANS response between toddlers with ASD and typical peers during baseline and, in particular, in response to a JA task. Despite the relatively small sample size of the population enrolled in this research, autonomic dysregulation can be seen among ASD subjects. Indeed, both SDNN and CV (normalized SDNN) were higher in ASD at baseline, indicating an increased HRV at rest, before the start of the task. In fact, SDNN is an index of HRV, indicating the variability of the HR during the whole duration of recording. Indeed, SDNN is mathematically equal to total power of spectral analysis, and so reflects all the cyclic components responsible for variability in the period of recording (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Consistently, both absolute LF and HF power, were higher at baseline in ASD, fostering the hypothesis toward an autonomic dysregulation of this population, already very early in childhood, and independently from the task. Thus, the higher SDNN and CV experienced by the children with ASD at baseline could suggest an abnormal global dysfunction of the ASN. Indeed, previous literature suggests that a higher HRV is not always an index of better functioning (Stein et al., 2005). The negative role of excessive high CV at baseline is confirmed by its positive correlation with the item "initiation joint attention" of the ADOS-G, which means that higher problems with the initiation joint attention behavior are correlated with higher CV at baseline. Notably, SDNN (but not CV) remains higher in ASD children compared with TD, during task meaning that the global dysfunction of ANS in these patients persists throughout the recording.

Literature regarding the activation of the ANS in ASD is somewhat inconsistent with some studies indicated an abnormally higher sympathetic, parasympathetic activity or finding no differences with TD (Klusek et al., 2015). In our study, the fact that the between-groups difference in LF is significant while HF is not, may suggest a prevalence of LF component. The higher LF experienced by the children with ASD at baseline could suggest a higher activation of the SNS, related to a somewhat higher anxiety state before the beginning of the task. This fact, indirectly consistent with the existing literature on this topic (Ming et al., 2005; Bal et al., 2010; Daluwatte et al., 2013; Porges et al., 2013; Kushki et al., 2014; Panju et al., 2015).

Focusing on the LF power, it is worth noting that the trend from baseline to task is significantly different between the two groups. Indeed, while in ASD subjects the LF power decreased from baseline to task, TD children displayed the opposite trend, with an increased value of LF during the task. This fact suggests a significantly increased activation of the SNS among TD children during JA, normally concerned with mental effort in attention-demanding tasks (see, for example, Beauchaine et al., 2001). Previous studies have shown that while TD subjects demonstrate increased sympathetic arousal during active tasks, individuals with ASD may not show this effect and could even display paradoxically increased parasympathetic activity compared with controls (Toichi and Kamio, 2003).

As a result, during the task, there is a higher LF in TD than in ASD although it does not reach significance. The positive role of increasing LF during the task, is confirmed by the negative correlation between LF during this phase and Internalizing item of the CBCL Scale I TD group.

In this research, however, RSA does not appear to be different between the two groups, nor between the two phases. RSA has been theoretically linked to social engagement (Porges et al., 2013), and are normally higher in TD children with respect to ASDs (Watson et al., 2012); however, our subjects, possibly due to the extremely young age or to the relatively small sample size, did not display any statistical difference. In general terms, RSA is an index of both respiratory signal and, under very specific conditions may sometimes partially reflect, or be a marker of, cardiac vagal tone (Grossman and Kollai, 2003).

According to Porges (2007), a greater basal RSA amplitude and task dependent RSA suppression, may be a protective factor reducing the risk of developing psychopathologies, particularly those associated with social behavior. However, the results obtained in the present work failed to demonstrate RSA variations either between groups and between phases, fostering the need for further investigations on larger cohorts.

Few studies have investigated changes in HRV in response to social events and stimuli in young children with ASD. Corona et al. (1998) reported that 3–5-year-old children with ASD

failed to show an HR decrease to the feigned distress of an examiner, as compared to children with developmental delays (DD). The same group reported that both young children with ASD and a matched group of children with DD (mean age: 4 years) showed decreased HR when viewing a video of babies laughing or crying, suggesting that the children with ASD were as attentive to the videotapes of the infants as was the DD group (Sigman et al., 2003). More recently, Watson et al. (2012) compared physiological responses of children with ASD (mean age: 35 months) to those of chronological age-matched typically developing children (mean age: 33 months) children during exposure to non-social and child-directed speech (CDS) stimuli. They found an increased HR in children with ASD compared to TD, even during periods of sustained looking at stimuli, in both non-social and CDS stimuli. This was interpreted as an overactive sympathetic system or underactive parasympathetic system, or both. The RSA was not different between groups during the task, similarly to our findings; however, the authors did not evaluate the changes compared to the baseline. In another study, the RSA of children with ASD and TD (age range: 2–6 years) was evaluated during a baseline period and a stranger approach paradigm. This study revealed differences in patterns of RSA response to social events in the two groups. Specifically, while children with TD showed a RSA response (decrease from baseline to task) in all the condition, children with ASD were more likely to show a response during the intrusive "proximal" stranger approach than during the initial and less intrusive entry of the stranger into the room (Sheinkopf et al., 2013). Given that RSA reflects social response and engagement with the environment, this finding suggests that a higher level of interaction is needed to elicit normative physiological responses in children with ASD. These results suggest that the ability to modulate cardiac activity of young children in response to a social task is very stimulus-specific. The partial discrepancies between the different studies, including our, indicate that experimental design, and in particular, the intensity, salience, or type of the stimuli can influence ANS response and between-subject differences. In our study, the ASD children, seem not to engage in the task as they do not show an increase of sympathetic activity as the comparison group. However, the RSA did not significantly change from baseline to task in both groups.

Considering our preliminary results and the literature evidences, we could hypothesize that, aside the autonomic dysregulation noticed already at baseline, the ASD subjects did not display the increased SNS activation during the task seen among TD children, demonstrating a lower degree of mental engagement during JA.

Taken together, these results suggest that the unobtrusive measurement of ANS response during JA task, which was seen to be feasible and well tolerated also by ASD toddlers, could represent an early marker of social dysfunction in ASD, contributing to a more objective diagnosis and to the definition of a more tailored treatment protocol. However, as above mentioned, the procedure here described could probably form the basis for future investigations on this specific population in this field.

Limitations and Future Developments

Some limitations need to be considered when interpreting the results of this study. First, the relatively small sample size should also be acknowledged, limiting the significance of the results we found. However, we should mention that the restricted age range of toddlers with ASD does not ease the recruitment of a numerous cohort. Second, we did not include a control group with a developmental quotient similar to that of the ASD group. The comparison with a TD group could prevent our result to be considered specific of ASD, and could be questioned whether the group effects reflect differences specifically due to ASD. Nevertheless, this limitation is reduced by the use of the nonverbal development quotient as a covariate in all between-subject comparisons. Finally, we evaluated ANS in response to videos made up of JA tasks, providing a partial assessment of JA as compared with a real-life situation; therefore, also the physiological response could be altered.

In the future, the findings of the study need to be replicated in a larger sample to prove the efficacy of the approach and to consolidate the results obtained. Larger samples will also allow for the evaluation of how different could be the physiological response of subgroups of children with ASD, i.e., high and low functioning children. As JA is an early marker of impairments in ASD, in a larger sample and even in young children it could be important to test the predictive value of ANS assessment for clinical outcomes in these groups. In addition, future studies could include multiple measures of the ANS, including additional HRV features (as the estimation of phasic contribution), GSR and respiration, using synchronized wearable sensors to evaluate relationships within and between components, and their relationships to social response during JA tasks.

CONCLUSIONS

In conclusion, in this study, we demonstrated the feasibility of using a wearable non-invasive technology for characterizing ANS response in toddlers with ASD during a social attention task. Our results possibly suggest autonomic dysregulation in ASD already at baseline. In addition, TD children showed an increased LF during the JA task, with an opposite trend with respect to ASD children. This result possibly demonstrates a different attitude toward JA, with a significantly higher mental effort performed by neurotypical children.

Both ASD and TD subjects, however, fail to exhibit a variation in RSA during JA, proving the necessity of future studies on larger cohorts. The results of this study foster the application of the proposed approach for evaluating physiological correlates of JA response in very young children and toddlers with ASD.

AUTHOR CONTRIBUTIONS

LB helped in data collection, guided in data and statistical analyses, and wrote the final version of the manuscript; AT performed the data and statistical analysis and made a manuscript draft; ZM helped in the data and statistical analysis;

MV contributed in the ECG signals analysis; LB, AN, and FM conceived the study; AN and CL participated in data collection; AN, CL, SC, and FF participated in the clinical assessment of the subjects; FM was responsible of recruitment and diagnosis of children; FM, MV, and CL contributed in the discussion and approval of the paper.

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Normative Values for Heart Rate Variability Parameters in School-Aged Children: Simple Approach Considering Differences in Average Heart Rate

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Background: Heart rate variability (HRV) analysis is a clinical tool frequently used to characterize cardiac autonomic status. The aim of this study was to establish normative values for short-term HRV parameters by considering their main determinants in school-aged children.

Methods: Five-minute electrocardiograms were taken from 312 non-athlete children (153 boys) at age of 6 to 13 years for computation of conventional time- and frequency-domain HRV parameters. Heart rate (HR), respiratory rate, age, body mass index, and sex were considered as their potential determinants. Multiple regression analysis revealed that HR was the principal predictor of all standard HRV indices. To develop their universal normative limits, standard HRV parameters were corrected for prevailing HR.

Results: The HRV correction for HR yielded the parameters which became independent on both sex and HR, and only poorly dependent on age (with small effect size). Normal ranges were calculated for both time- and frequency-domain indices (the latter computed with either fast Fourier transform and autoregressive method). To facilitate recalculation of standard HRV parameters into corrected ones, a calculator was created and attached as a **Supplementary Material** that can be downloaded and used for both research and clinical purposes.

Conclusion: This study provides HRV normative values for school-aged children which have been developed independently of their major determinants. The calculator accessible in the **Supplementary Material** can considerably simplify determination if HRV parameters accommodate within normal limits.

Keywords: heart rate, heart rate variability, heart rate correction, children and adolescents, normative values, reference values, autonomic nervous system, autonomic cardiac control

INTRODUCTION

Since the early 1970s, when power spectral analysis was applied to explore the physiological basis of intermittent variations in heart rate (HR) (Hyndman et al., 1971; Chess et al., 1975; Akselrod et al., 1981; Billman, 2011; Ernst, 2017b), a large number of studies addressing heart rate variability (HRV) have been published (Xhyheri et al., 2012). Reduced HRV corresponds to the autonomic nervous system (ANS) imbalance and may be associated with worse prognosis (particularly increased mortality) in various disease states among adults (Kleiger et al., 1987; Dekker et al., 2000; Stein et al., 2005; Thayer et al., 2010; Ernst, 2017a). Also in children, depressed HRV may be related to some cardiac (Massin and von Bernuth, 1998; Heragu and Scott, 1999; Bakari et al., 2013) and non-cardiac disorders (Chessa et al., 2002; Birch et al., 2012). However, to diagnose the ANS imbalance normative or reference values for HRV indices need to be established. Nevertheless, only a limited number of studies reported such normative/reference HRV values in pediatric populations (Goto et al., 1997; Massin and von Bernuth, 1997; Umetani et al., 1998; Silvetti et al., 2001; Rêkawek et al., 2003; Longin et al., 2009; Michels et al., 2013; Seppälä et al., 2014; Jarrin et al., 2015; Sharma et al., 2015; Bobkowski et al., 2017). Usually, authors presented their normal values for children by categorizing them according to age and/or sex (Massin and von Bernuth, 1997; Silvetti et al., 2001; Rêkawek et al., 2003; Sharma et al., 2015), however, HRV parameters are also significantly associated with other factors, like HR (Goto et al., 1997; Massin and von Bernuth, 1997; Jarrin et al., 2015), respiration (Grossman and Taylor, 2007; Beda et al., 2014; Quintana et al., 2016a,b; Shader et al., 2017; Shaffer and Ginsberg, 2017), physical activity (Oliveira et al., 2017), or weight status (Eyre et al., 2014). Since HRV is primarily HR dependent (Fleiss et al., 1992; Goto et al., 1997; Massin and von Bernuth, 1997; Sacha and Grzeszczak, 2001; Sacha and Pluta, 2005a,b; Sacha and Pluta, 2008; Burr et al., 2006; Nieminen et al., 2007; Billman, 2013; Sacha, 2013, 2014a,b,c; Sacha et al., 2013a,b,c; Monfredi et al., 2014; Billman et al., 2015; Gąsior et al., 2015; Jarrin et al., 2015), any alteration in average HR automatically changes HRV. Consequently, a comparison of HRV corresponding to different HR may be biased (Sacha and Grzeszczak, 2001; Nieminen et al., 2007; Sacha and Pluta, 2008). Mathematical methods removing the HRV dependence on HR (i.e., the HRV correction for prevailing HR) (Hayano et al., 1990, 1991; Sacha and Grzeszczak, 2001; Sacha and Pluta, 2008; Sacha et al., 2013b,c; Monfredi et al., 2014; Estévez-Báez et al., 2015; van Roon et al., 2016) should allow to draw objective conclusions when comparing HRV associated with various HR (Sacha, 2013, 2014a,b,c; Monfredi et al., 2014; Billman et al., 2015). So far, only one study dealing with normal ranges of HRV employed the HRV correction for HR, however, authors presented normal values only for two time domain HRV markers derived from 10-s ECGs (van den Berg et al., 2018). Recently, researchers applying such a correction have demonstrated a significant interaction between HRV and HR in healthy pediatric populations (Gąsior et al., 2015; Bjelakovic et al., 2017; Herzig et al., 2017). It has been shown that while standard HRV may remain constant in different age subgroups, corrected HRV decreases with age along with a decrease in average HR.

Another important factor, usually not accounted for in HRV normative studies but significantly influencing both HR and HRV, is breathing (Quintana et al., 2016a; Shader et al., 2017). The respiratory rate declines from birth to early adolescence (Fleming et al., 2011), and consequently such changes may influence HRV (Billman, 2011; Quintana and Heathers, 2014; Quintana et al., 2016a,b; Laborde et al., 2017).

The aims of the present study were to determine the main determinants of HRV in healthy school-aged children, and by incorporating the significant determinants to define normative values for both time- and frequency-domain HRV indices.

MATERIALS AND METHODS

Study Population

The whole study group consisted of 346 children of both sexes. The inclusion criteria to this study were as follows: (i) age between 6 and 13 years, (ii) absence of diseases and/or regular use of medications affecting the cardiopulmonary system and/or interfering with the autonomic nervous system, and (iii) not being an active athlete in any sports. The parents/legal guardians were interviewed about children's diseases and/or medications (the school health records concerning health status were additionally verified). Fifteen subjects were excluded from the analysis due to suspicion of cardiac/non-cardiac diseases. The group participated in an earlier study, its details and description of the procedures imposed before or during the proper electrocardiographic examinations have been published elsewhere (Gąsior et al., 2015). Since there are data indicating an association of cardiac autonomic function with physical activity in children and adolescents (Oliveira et al., 2017), we excluded 19 active athletes (Araújo and Scharhag, 2016) from the present study providing normative values. Consequently, 312 healthy Caucasian children of both sexes between the ages of 6 and 13 years were enrolled in the final analysis. The group was divided into four subgroups according to age: (I) $6 \leq$ age < 8 years; (II) $8 \leq$ age < 10 years; (III) $10 \leq$ age < 12 years; and (IV) $12 \leq$ age < 14 years. This classification is in accordance with other studies where participants younger than 10 years were considered as children and those between 10 and 14 years as preadolescents or early adolescents (Frigerio et al., 2006; Cysarz et al., 2011; Varlinskaya et al., 2013). The study was approved by the University Bioethical Committee and followed the rules and principles of the Helsinki Declaration, as well as all parents or legal guardians gave their informed written consent.

Body Mass Status Measurement

The body mass status was measured using Body Mass Index (BMI) defined as body mass in kilograms divided by height in meters squared. BMI is widely used for assessing the weight status of pediatric participants since it can identify those who are overweight, at risk of being overweight,

or underweight based on age and sex (Barlow and Dietz, 1998).

Electrocardiographic (ECG) Recordings and HRV Analysis

Twelve-lead, 6-min ECG recordings (sampling frequency = 1000 Hz) were performed using a portable PC with integrated software system (Custo cardio 100 12-channel PC ECG system; Custo med GmbH, Ottobrunn, Germany) during regular school days between 8 a.m. and 2 p.m. in a supine position. For heart rate stabilization, children were placed in a supine position for 5 min before the examination. All ECG recordings were preprocessed before HRV analysis, i.e., they were visually inspected for potential non-sinus or aberrant beats and such erroneous beats were properly corrected (interpolation of degree zero) (Peltola, 2012). Time- and frequency-domain HRV analyses were performed from 5-min ECG recordings by using Kubios HRV Standard 3.0.2 software (University of Eastern Finland, Kuopio, Finland) (Tarvainen et al., 2014, 2017). Standard deviation of R-R intervals (SDNN), root mean square of successive R-R interval differences (RMSSD), and pNN50, which denotes percent of R-R intervals differing >50 ms from the preceding one, were determined. Before calculating spectral HRV parameters, smoothness priors based detrending approach was applied (smoothing parameter, Lambda value = 500) (Tarvainen et al., 2002), and then R-R interval series were transformed to evenly sampled time series with 4-Hz resampling rate. The detrended and interpolated R-R interval series were used to compute HRV spectra by employing fast-Fourier-transform (FFT) with Welch's periodogram method (300 s window width without overlap) and by using the autoregressive model (AR) with model order set at 16 (Boardman et al., 2002). The following spectral components were distinguished: very low frequency (VLF, 0–0.04 Hz), low frequency (LF, 0.04–0.15 Hz), high frequency (HF, 0.15–0.50 Hz), and total power [(in two versions, i.e., TP_1, 0–0.5 Hz (VLF+LF+HF) and TP_2, 0.04–0.50 Hz (LF+HF)] in absolute units (ms^2), as well as nLF, nHF in normalized units (nu) (Task Force, 1996).

ECG Derived Respiration

The respiratory rate (RespRate) was calculated from ECG recordings according to Sinnecker et al. (2014). The time series of the QRS amplitude for all 12 ECG leads were calculated and then local maxima (i.e., data points with values greater than both the preceding and the following data point) were established. Then, the mean interval between consecutive local maxima for each time series was computed and the reciprocal value of the mean maximum-to-maximum interval was obtained. The median of these reciprocal values over all-time series represented RespRate used in this analysis (Sinnecker et al., 2014).

HRV Correction

Since HRV is dependent on HR due to both physiological (i.e., the autonomic nervous system influence) and mathematical (i.e., the inverse non-linear relationships between HR and R-R interval) reasons, it is hard to ascertain if clinical significance of HRV comes from the variability or from HR (Sacha and Pluta, 2008) – it is even more important in populations where average HR is changing, like in children during their grow and development. Therefore, to investigate the "objective" variability, one should get rid of the HR impact on HRV. To do so, one should correct the HRV for the prevailing HR. The correction procedure employed in this study relied on the division or multiplication of standard HRV indices by different powers of their corresponding mean R-R interval (mRR) – the higher HRV dependence on HR, the higher power of mRR must be used to get rid of this dependence. Specifically, if HRV parameters revealed negative relation with HR (i.e., SDNN, RMSSD, pNN50, VLF, LF, HF, TP_1, TP_2, and nHF), they should be divided by the suitable power of mRR in order to become HR independent, however, if the HRV parameters are positively related with HR (i.e., nLF and LF/HF), they must be multiplied by the appropriate power of mRR – this way we were able to remove both physiological and mathematical HRV dependence on HR (Sacha et al., 2013b,c).

Statistical Analysis

The Kolmogorov–Smirnov test was used to assess the normality of the data distribution. Normally distributed values were presented as mean ± standard deviation, but others as median and interquartile range (IQR). Accordingly, Pearson's correlation coefficient (r) or Spearman's rank correlation coefficient (R) were used to assess the relationship between variables, and Student's *t*-test or non-parametric Mann–Whitney test were employed to determine differences. The Pearson's chi-squared test was applied to determine differences between variables prevalence in multiple groups. Values of the standard and corrected HRV parameters were compared between four age subgroups by using Kruskal–Wallis (K-W) test and analysis of variance (ANOVA), respectively. Multiple regression analysis was carried out to identify independent determinants of standard and corrected HRV parameters – variables with a skewed distribution were logarithmically transformed (ln). To avoid multicollinearity, redundant variables were removed from the multivariate regression models in the case of pairwise correlations between continuous variables (Slinker and Glantz, 2008). The effect size was measured by calculating Cohen's f^2 within a multiple regression model (Selya et al., 2012). The f^2 were calculated for a combined prediction of the model, and Cohen's f^2 variation was computed to measure the local effect size (Cohen, 1992; Selya et al., 2012). According to Cohen's guidelines, $f^2 \geq 0.02$, $f^2 \geq 0.15$, and $f^2 \geq 0.35$ represent small, medium, and large effect sizes, respectively (Cohen et al., 2003). The threshold probability of $p < 0.05$ was taken as the level of statistical significance. Since all HRV parameters were not normally distributed, normative ranges have been given as medians with the 5th and 95th percentiles. Statistical calculations were performed by using the software STATISTICA 10 (StatSoft, Inc., Software, Tulsa, OK, United States). Graphs were created with GraphPad PRISM® Version 5.0 (GraphPad Software, Inc., San Diego, CA, United States) and Microsoft Office Excel 2007 (Microsoft Corporation, Silicon Valley, CA, United States).

RESULTS

The group of 312 healthy non-athlete children (153 ♂, 159 ♀) at age of 6 to 13 years (median: 10.1 years, IQR: 8.4–11.8 years) were analyzed in the present study. The group's characteristics were: median body mass, 34.1 kg (IQR: 27.7–45.1 kg); median height, 140.0 cm (IQR: 129.5–152.5 cm); median BMI, 17.3 kg/m^2 (IQR: 15.7–20.1 kg/m^2); mean HR, 84.4 ± 10.6 bpm (range: 54.7–113.8 bpm); and mean respiratory rate (RespRate), 22.7 ± 3.2 bpm (range: 12.4–30.7 bpm).

Comparing with boys, girls presented significantly higher HR, lower values of all time-domain HRV parameters, i.e., SDNN, RMSSD, pNN50, and the following frequency-domain parameters (calculated with both FFT and AR): VLF, LF, TP_1 (VLF+LF+HF), TP_2 (LF+HF) ($p < 0.05$ for all). Boys and girls did not differ in RespRate ($p = 0.64$), BMI ($p = 0.71$), and the following spectral parameters: HF, LF/HF, nLF, and nHF ($p > 0.05$ for all).

Heart rate, RespRate, age, BMI and sex were initially chosen as potential determinants of standard HRV parameters (**Table 1** presents their correlations with HRV). All these variables were associated with each other with one exception, i.e., there was no significant correlation between RespRate and BMI (**Figure 1**). Since the association between RespRate and HR was stronger than between RespRate and HRV, as well as, the association between BMI and age was stronger than between BMI and HRV, (**Figure 1** and **Table 1**), both RespRate and BMI were considered to be redundant for the multivariate analysis (collinearity tests confirmed this redundancy). Accordingly to the statistical assumptions of the multiple regression analysis (Kraha et al., 2012; Ray-Mukherjee et al., 2014) and taking into account the practicality of normative data in a clinical context, the following variables were included in the regression analysis: HR, age, and sex. **Table 2** exhibits results of this analysis for time- and frequency-domain HRV parameters calculated with FFT – the results for HRV spectra estimated with AR were similar and are presented in **Supplementary Table S1**. The models accounted for 9–60% of the entire HRV variance, but importantly, HR was the strongest predictor for all analyzed standard HRV parameters. HR was the most powerful determinant in the whole group (**Table 2** and **Supplementary Table S1**) and also within the age subgroups: 6–7, 8–9, 10–11, and 12–13 years (**Supplementary Tables S2–S9**).

Since HR was the strongest predictor for all standard HRV parameters, in order to calculate the normative values, the overall study group was classified into four quartile subgroups based on HR value: 1st quartile (Q1: <25%), 54.7–77.8 bpm; 2nd quartile (Q2: 25–50%), >77.8–84.3 bpm; 3rd quartile (Q3: 50–75%), >84.3–92.0 bpm; and 4th quartile (Q4: >75%), >92.0–113.8 bpm. The number of participants equaled to 78 in each subgroup: Q1 (*male*/*female*), 45/33; Q2, 41/37; Q3, 32/46; and Q4, 35/43. There was no significant difference in sex distribution in these subgroups (Pearson's Chi-squared = 5.27, $p = 0.15$). There were statistically significant differences in all standard HRV parameters between the HR quartile subgroups (results of K-W test ranged between 26.6 and 160.8 with $p < 0.001$ for all). Normative values of standard time- and frequency-domain HRV parameters according to HR quartiles are presented in **Table 3**.

TABLE 1 | Correlations of standard time- and frequency-domain HRV parameters with heart rate, respiratory rate, age, and body mass index.

Standard HRV parameter	HR [bpm]		RespRate [breaths/min]		Age [years]		BMI [kg/m^2]	
	R	*p*	*R*	*p*	*R*	*p*	*R*	*p*
SDNN [ms]	−0.64		−0.41		−0.03	0.61	−0.04	0.52
RMSSD [ms]	−0.72		−0.37		< −0.01	0.99	−0.04	0.45
pNN50 [%]	−0.75		−0.36		0.02	0.72	−0.04	0.54
$_{FFT}$ VLF [ms^2]	−0.42		−0.22		−0.07	0.23	0.04	0.50
$_{FFT}$ LF [ms^2]	−0.53		−0.31		−0.04	0.49	< 0.01	0.97
$_{FFT}$ HF [ms^2]	−0.60		−0.44		−0.06	0.29	−0.07	0.22
$_{FFT}$ TP_1 (VLF+LF+HF) [ms^2]	−0.60		−0.41		−0.06	0.28	−0.04	0.46
$_{FFT}$ TP_2 (LF+HF) [ms^2]	−0.60		−0.41		−0.06	0.31	−0.05	0.42
$_{FFT}$ LF/HF	0.25		0.26		0.05	0.36	0.10	0.08
$_{FFT}$ nLF [nu]	0.25	<0.001	0.26	<0.001	0.05	0.36	0.10	0.08
$_{FFT}$ nHF [nu]	−0.25		−0.26		−0.05	0.36	−0.10	0.08
$_{AR}$ VLF [ms^2]	−0.56		−0.33		−0.08	0.19	−0.02	0.68
$_{AR}$ LF [ms^2]	−0.56		−0.34		−0.02	0.77	0.01	0.81
$_{AR}$ HF [ms^2]	−0.63		−0.45		−0.03	0.57	−0.06	0.28
$_{AR}$ TP^1 (VLF+LF+HF) [ms^2]	−0.63		−0.43		−0.03	0.64	−0.03	0.57
$_{AR}$ TP^2 (LF+HF) [ms^2]	−0.63		−0.43		−0.02	0.67	−0.03	0.55
$_{AR}$ LF/HF	0.26		0.25		0.05	0.36	0.10	0.06
$_{AR}$ nLF [nu]	0.26		0.25		0.05	0.36	0.10	0.06
$_{AR}$ nHF [nu]	−0.26		−0.25		−0.05	0.37	−0.10	0.06

FFT denotes that a given HRV parameter was calculated by using Fast Fourier Transform, while AR indicates that the HRV spectrum was calculated with autoregressive method. R, Spearman's rank correlation coefficient.

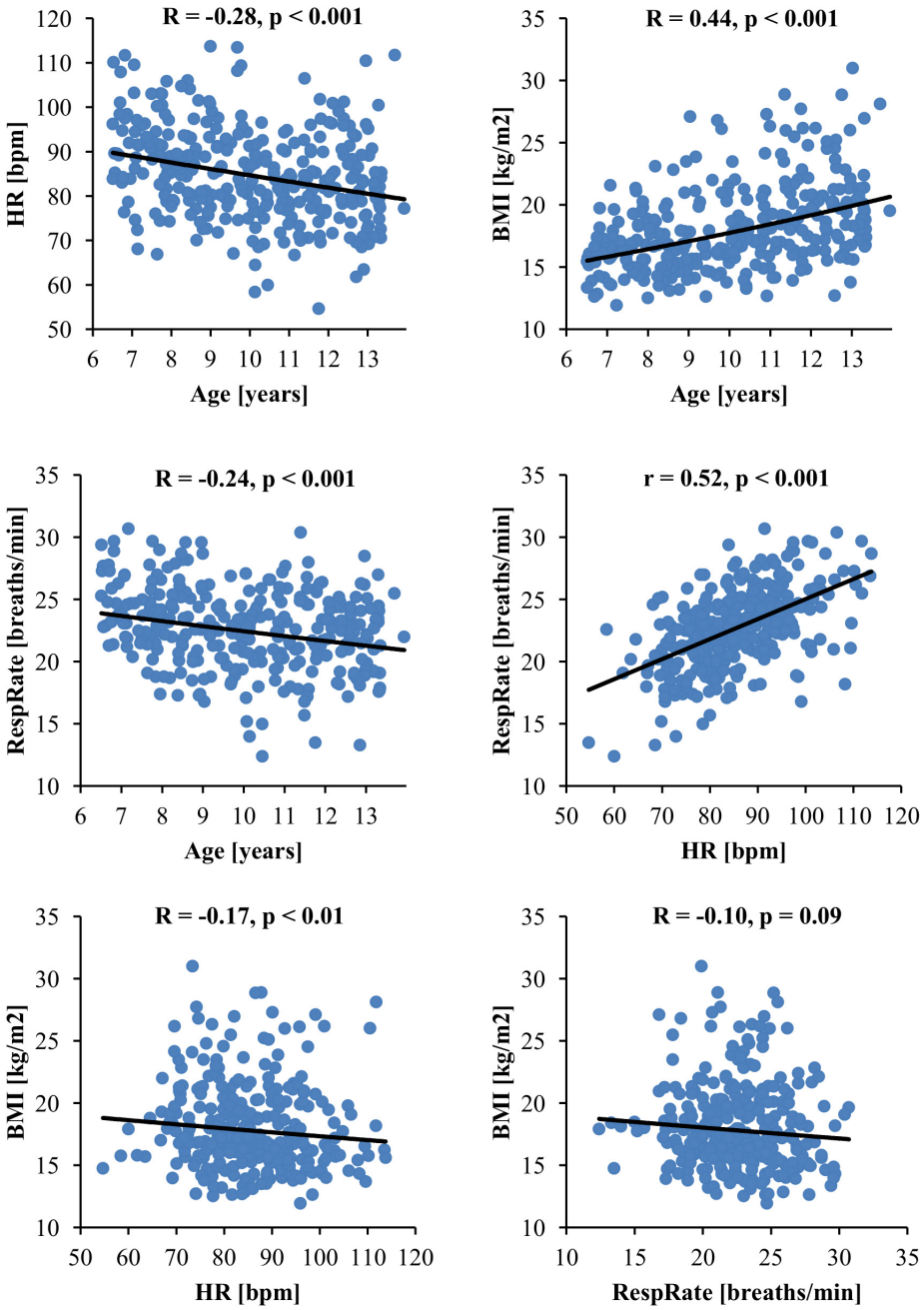

FIGURE 1 | Association between variables selected as potential independent predictors of standard HRV parameters. BMI, body mass index; HR, heart rate; HRV, heart rate variability; RespRate, respiratory rate; R, Spearman's rank correlation coefficient; r, Pearson's correlation coefficient.

However, there was a significant overlap in the normative ranges between consecutive HR quartile subgroups which makes such data impractical. Therefore, considering the fact that HR was the most influential factor determining all standard HRV parameters, we excluded its impact by correcting the standard HRV parameters for their prevailing HR. This way we were able to get normative values for so called corrected HRV, i.e., the variability independent on average HR. Indeed, standard time-domain HRV parameters lost their dependence on HR after dividing SDNN, RMSSD, and pNN50 by mRR to the power: 2.2, 3.0, and 5.0, respectively. In spectral analysis, VLF obtained by FFT and AR lost its dependence on HR after dividing by mRR to the power 3.0 and 4.0, respectively. Other frequency-domain HRV indices lost their association with HR after dividing LF, HF, TP_1 (VLF+LF+HF), TP_2 (LF+HF), and nHF by mRR to the power: 4.0, 5.0, 5.0, 5.0, and 0.5, respectively (for both FFT and AR). The nLF and LF/HF (calculated with either FFT and AR) stopped being dependent on HR after multiplying by mRR to the

TABLE 2 | Determinants of standard time- and frequency-domain (calculated with FFT) HRV parameters.

Standard HRV parameter	Determinant	Parameters of multiple regression analysis					
		β	*p*	Partial correlation	Multiple R^2	*F*-test	*p*
SDNN (ln)	HR	−0.73	< 0.001	−0.70	0.50	102.3	<0.001
	Age (ln)	−0.23	< 0.001	−0.30			
	Sex	0.03	0.54	0.04			
RMSSD (ln)	HR	−0.81	< 0.001	−0.77	0.60	153.8	<0.001
	Age (ln)	−0.24	< 0.001	−0.35			
	Sex	0.01	0.74	0.02			
pNN50 (ln)	HR	−0.77	< 0.001	−0.72	0.53	113.0	<0.001
	Age (ln)	−0.23	< 0.001	−0.30			
	Sex	−0.02	0.59	−0.03			
VLF (ln)	HR	−0.49	< 0.001	−0.47	0.24	31.9	<0.001
	Age (ln)	−0.19	< 0.001	−0.21			
	Sex	0.05	0.30	0.06			
LF (ln)	HR	−0.61	< 0.001	−0.58	0.36	57.3	<0.001
	Age (ln)	−0.20	< 0.001	−0.23			
	Sex	0.08	0.09	0.10			
HF (ln)	HR	−0.70	< 0.001	−0.67	0.46	85.9	<0.001
	Age (ln)	−0.26	< 0.001	−0.32			
	Sex	< 0.01	0.88	< 0.01			
TP_1 (VLF+LF+HF) (ln)	HR	−0.69	< 0.001	−0.67	0.45	85.4	<0.001
	Age (ln)	−0.24	< 0.001	−0.30			
	Sex	0.03	0.43	0.05			
TP_2 (LF+HF) (ln)	HR	−0.70	< 0.001	−0.67	0.46	86.1	<0.001
	Age (ln)	−0.24	< 0.001	−0.30			
	Sex	0.03	0.46	0.04			
LF/HF (ln)	HR	0.31	< 0.001	0.29	0.09	10.4	<0.001
	Age (ln)	0.15	< 0.01	0.15			
	Sex	0.10	0.08	0.10			
nLF	HR	0.31	< 0.001	0.30	0.10	10.8	<0.001
	Age (ln)	0.16	< 0.01	0.15			
	Sex	0.10	0.07	0.10			
nHF	HR	−0.31	< 0.001	−0.30	0.10	10.8	<0.001
	Age (ln)	−0.15	< 0.01	−0.15			
	Sex	−0.10	0.07	−0.10			

power 1.0 (Sacha et al., 2013b). Correlation coefficients between HR and all corrected HRV parameters were not statistically significant and ranged between −0.09 and 0.08 ($p > 0.10$ for all).

In the multiple regression analysis, age turned out to be the strongest determinant of the corrected time- and frequency-domain HRV parameters calculated with FFT (**Table 4**) – the results were the same when HRV spectra were estimated with AR (the AR data are presented in **Supplementary Table S10**). However, the calculated models accounted for only 2–9% of the whole variance of the corrected HRV indices. The age contribution to this model was quite low (i.e., β-value ranged between −0.13 and −0.29), and the effect size for the combined model, as well as for the variables taken individually (age and sex) was small ($f^2 \leq 0.091$ for all). Therefore, practically there was no reason to establish normative values according to age, and we calculated normal limits for the overall study group (**Table 5**). Of note, due to division by high powers of mRR, the numbers in **Table 5** are long (i.e., they present many zeros after the decimal place), consequently calculations and interpretations of such results may be uninterpretable in clinical situations, hence in the **Supplementary Material** one may find the Excel sheet calculator which recalculates standard HRV parameters into corrected ones and determines whether a given value of corrected HRV parameter is within normal limits (**Supplementary Table S11**, Sheet "Corrected HRV Calculator").

DISCUSSION

The aim of the present study was to define, by incorporating the significant determinants, normative values for HRV indices in healthy children. HRV is changing during childhood and adolescence which may reflect developmental alterations in the ANS activity. Thus, in order to follow these changes, normal

TABLE 3 | Normative standard time- and frequency-domain HRV parameters values according to heart rate quartiles are presented as median, 5th–95th percentiles.

Standard HRV parameter	Q1	Q2	Q3	Q4
SDNN [ms]	75 (38–118)	56 (29–95)	43 (25–80)	35 (16–54)
RMSSD [ms]	86 (42–137)	65 (31–107)	44 (22–89)	33 (15–55)
pNN50 [%]	54 (22–70)	42 (11–63)	23 (2–54)	10 (0–30)
$_{FFT}$ VLF [ms^2]	102 (30–389)	66 (19–220)	47 (13–138)	41 (8–216)
$_{FFT}$ LF [ms^2]	1479 (328–5162)	705 (162–3286)	546 (236–2562)	421 (75–1193)
$_{FFT}$ HF [ms^2]	3269 (681–9485)	1808 (396–5679)	912 (265–4647)	527 (96–1956)
$_{FFT}$ TP_1 (VLF+LF+HF) [ms^2]	5096 (1178–13023)	2750 (777–8791)	1544 (563–6671)	1079 (222–2959)
$_{FFT}$ TP_2 (LF+HF) [ms^2]	4994 (1129–12787)	2703 (760—-8608)	1493 (528–6558)	1043 (206–2924)
$_{FFT}$ LF/HF	0.48 (0.17–1.31)	0.54 (0.12–1.42)	0.67 (0.24–1.97)	0.87 (0.21–2.45)
$_{FFT}$ nLF [nu]	32 (15–57)	35 (11–59)	40 (20–66)	46 (18–71)
$_{FFT}$ nHF [nu]	68 (43–86)	65 (41–89)	60 (34–81)	54 (29–82)
$_{AR}$ VLF [ms^2]	205 (68–516)	122 (32–397)	88 (34–284)	61 (18–191)
$_{AR}$ LF [ms^2]	1411 (376–4547)	828 (146–2702)	559 (195–2029)	387 (92–1216)
$_{AR}$ HF [ms^2]	3063 (717–5454)	1939 (396–6127)	988 (261–4325)	576 (85–1706)
$_{AR}$ TP_1 (VLF+LF+HF) [ms^2]	5061 (1444–12919)	2936 (791–8646)	1778 (580–6636)	1118 (202–2774)
$_{AR}$ TP_2 (LF+HF) [ms^2]	4826 (1248–12277)	2800 (734–8404)	1662 (542–6502)	1015 (184–2638)
$_{AR}$ LF/HF	0.44 (0.17–1.32)	0.55 (0.15–1.19)	0.61 (0.26–1.83)	0.73 (0.23–2.29)
$_{AR}$ nLF [nu]	30 (14–57)	35 (13–54)	38 (20–65)	42 (19–70)
$_{AR}$ nHF [nu]	69 (43–86)	65 (46–87)	62 (35–79)	58 (30–81)

FFT denotes that a given HRV parameter was calculated by using Fast Fourier Transform, while AR indicates that the HRV spectrum was calculated with autoregressive method. HR quartiles for overall study group: (Q1), 54.7–77.8 bpm; (Q2), >77.8–84.3 bpm; (Q3), >84.3–92.0 bpm; (Q4), >92.0–113.8 bpm.

TABLE 4 | Determinants and Cohen's f^2 indexes for corrected time- and frequency-domain (calculated with FFT) HRV parameters.

Corrected HRV parameter	Det.	Parameters of multiple regression analysis						Cohen's f^2	
		β	*p*	PC	M. R2	*F*-test	*p*	Loc.	Comb.
corr-SDNN	Age (ln)	−0.24	< 0.001	−0.24	0.06	10.6	< 0.001	0.063	0.063
	Sex	0.07	0.21	0.07				0.005	
corr-RMSSD	Age (ln)	−0.28	< 0.001	−0.28	0.08	13.9	< 0.001	0.086	0.086
	Sex	0.06	0.27	0.06				0.004	
corr-pNN50	Age (ln)	−0.28	< 0.001	−0.28	0.08	12.8	< 0.001	0.083	0.083
	Sex	0.01	0.80	0.01				0.001	
corr-VLF	Age (ln)	−0.20	< 0.001	−0.20	0.04	6.3	< 0.01	0.041	0.039
	Sex	0.03	0.62	0.03				0.001	
corr-LF	Age (ln)	−0.20	< 0.001	−0.21	0.06	9.1	< 0.001	0.044	0.044
	Sex	0.12	< 0.05	0.12				0.014	
corr-HF	Age (ln)	−0.20	< 0.001	−0.20	0.04	6.7	< 0.01	0.044	0.044
	Sex	0.01	0.95	0.01				0.001	
corr-TP_1 (VLF+LF+HF)	Age (ln)	−0.25	< 0.001	−0.25	0.06	10.2	< 0.001	0.065	0.065
	Sex	0.03	0.54	0.04				0.001	
corr-TP_2 (LF+HF)	Age (ln)	−0.24	< 0.001	−0.24	0.06	9.8	< 0.001	0.063	0.063
	Sex	0.03	0.54	0.04				0.001	
corr-LF/HF	Age (ln)	0.13	< 0.05	0.13	0.02	3.3	< 0.05	0.017	0.017
	Sex	0.06	0.26	0.06				0.004	
corr-nLF	Age (ln)	0.15	< 0.01	0.15	0.03	5.4	< 0.01	0.024	0.024
	Sex	0.11	0.06	0.11				0.011	
corr-nHF	Age (ln)	−0.15	< 0.01	−0.15	0.03	5.0	< 0.01	0.024	0.024
	Sex	−0.10	0.08	−0.11				0.008	

*Abbreviations: Det., determinant; PC, partial correlation; M. R2, multiple R2; Loc., local; Comb., combined. Corrected HRV parameters were calculated as follows: corr-SDNN, SDNN/mRR^2.2; corr-RMSSD, RMSSD/mRR^3.0; corr-pNN50, pNN50/mRR^5.0; FFT corr-VLF, VLF/mRR^3.0; AR corr-VLF, VLF/mRR^4.0; for both FFT and AR: corr-LF, LF/mRR^4.0; corr-HF, HF/mRR^5.0; corr-TP_1, TP_1/mRR^5.0; corr-TP_2, TP_2/mRR^5.0; corr-LF/HF, LF/HF*mRR^1.0; corr-nLF, nLF*mRR^1.0; and corr-nHF, nHF/mRR^0.5.*

TABLE 5 | Normative values for corrected HRV parameters for the whole study group.

Corrected HRV parameters	Normative values		
	Median	5th percentile	95th percentile
corr-SDNN [$ms^{-1.2}$]	2.6E-05	1.5E-05	4.6E-05
corr-RMSSD [ms^{-2}]	1.4E-07	7.6E-08	2.6E-07
corr-pNN50 [$\%/ms^{5}$]	1.4E-13	2.0E-14	3.0E-13
$_{FFT}$ corr-VLF [ms^{-1}]	1.7E-07	4.4E-08	5.5E-07
$_{FFT}$ corr-LF [ms^{-2}]	2.9E-09	7.5E-10	9.8E-09
$_{FFT}$ corr-HF [ms^{-3}]	6.8E-12	1.7E-12	2.5E-11
$_{FFT}$ corr-TP_1 (VLF+LF+HF) [ms^{-3}]	1.2E-11	3.6E-12	3.8E-11
$_{FFT}$ corr-TP_2 (LF+HF) [ms^{-3}]	1.1E-11	3.3E-12	3.7E-11
$_{FFT}$ corr-LF/HF [ms]	4.5E+02	1.5E+02	1.2E+03
$_{FFT}$ corr-nLF [nu*ms]	2.7E+04	1.3E+04	4.5E+04
$_{FFT}$ corr-nHF [$nu/ms^{0.5}$]	2.3E+00	1.4E+00	3.1E+00
$_{AR}$ corr-VLF [ms^{-2}]	4.3E-10	1.4E-10	1.2E-09
$_{AR}$ corr-LF [ms^{-2}]	2.7E-09	7.9E-10	8.8E-09
$_{AR}$ corr-HF [ms^{-3}]	7.3E-12	1.6E-12	2.7E-11
$_{AR}$ corr-TP_1 (VLF+LF+HF) [ms^{-3}]	1.2E-11	3.7E-12	4.0E-11
$_{AR}$ corr-TP_2 (LF+HF) [ms^{-3}]	1.1E-11	3.4E-12	3.8E-11
$_{AR}$ corr-LF/HF [ms]	4.1E+02	1.4E+02	1.1E+03
$_{AR}$ corr-nLF [nu*ms]	2.6E+04	1.2E+04	4.3E+04
$_{AR}$ corr-nHF [$nu/ms^{0.5}$]	2.4E+00	1.5E+00	3.1E+00

*FFT denotes that a given HRV parameter was calculated by using Fast Fourier Transform, while AR indicates that the HRV spectrum was calculated with autoregressive method. Corrected HRV parameters were calculated as follows: corr-SDNN, SDNN/mRR^2.2; corr-RMSSD, RMSSD/mRR^3.0; corr-pNN50, pNN50/mRR^5.0; FFT corr-VLF, VLF/mRR^3.0; AR corr-VLF, VLF/mRR^4.0; for both FFT and AR: corr-LF, LF/mRR^4.0; corr-HF, HF/mRR^5.0; corr-TP_1, TP_1/mRR^5.0; corr-TP_2, TP_2/mRR^5.0; corr-LF/HF, LF/HF*mRR^1.0; corr-nLF, nLF*mRR^1.0; and corr-nHF, nHF/mRR^0.5.*

values for HRV parameters need to be established. This study provides such normative quantities for short-term (i.e., 5 min) time- and frequency-domain HRV parameters based on a group of 312 school-aged children, i.e., at age of 6–13 years.

Since HRV is under influence of a number of factors, we conducted the multiple regression analysis which revealed that HR is the strongest predictor for all standard HRV parameters in the overall study group and in each analyzed age subgroups (**Table 2** and **Supplementary Tables S1–S9**). Age was the second independent predictor but accounted only for a small percentage of the entire HRV variance. Interestingly, in the multivariate analysis sex did not independently determine HRV in our study population (**Table 2** and **Supplementary Table S1**). The reason for this possibly comes from the observation that boys usually present significantly lower HR than girls and therefore sex differences in HRV may result from differences in HR. When considering HR, we could ignore differences between the sexes and establish normative HRV values only for subgroups categorized according to the quartiles of HR (**Table 3**). However, among such specified subgroups, confidence intervals significantly overlapped for most of HRV parameters, which may contest practicality of this approach. Hence, we calculated HRV independent on HR by correcting standard HRV indices for prevailing HR. In this method, we obtained so-called corrected HRV parameters, which reflect "objective" variability regardless whatever the average HR is. In the multiple regression analysis, age was significant predictor for such corrected HRV indices (**Table 4** and **Supplementary Table S10**), nevertheless, its contribution to the whole variance of HRV was small, i.e., the models only accounted for up to 9% of the HRV variance and the age role in these models was modest (β values ranged between −0.29 and 0.13) as well as its effect size turned out to be small ($f^2 \leq 0.091$) (**Table 4** and **Supplementary Table S10**). Therefore, we omitted age impact in these 6 to 13-year-old children and established normal limits for the entire study group (**Table 5**). The calculation of corrected HRV parameters and their assessment become simpler by using the file provided in the **Supplementary Material**, which recalculates standard HRV indices into corrected ones and automatically determines whether they accommodate within normal limits (**Supplementary Table S11**, Sheet "Corrected HRV Calculator").

The influence of HR on HRV in children has also been recognized in other studies (Goto et al., 1997; Massin and von Bernuth, 1997; Jarrin et al., 2015; Bjelakovic et al., 2017; Herzig et al., 2017). Indeed, Jarrin et al. (2015), accounted the observation that HR is the strongest factor determining HRV and presented their normative values adjusted for HR, however, only in participants with a very narrow age range, i.e., 9–11 years (mean ± SD: 10.2 ± 0.3 years) (Jarrin et al., 2015). Yet, the problem of the strong association between HR and HRV can simply be resolved by correcting HRV parameters for prevailing HR (Sacha and Grzeszczak, 2001; Sacha and Pluta, 2005a,b, 2008; Sacha, 2013, 2014a,b,c; Sacha et al., 2013a,b,c, 2014; Gąsior et al., 2016) and this method turns out to be feasible and convenient also in children populations (Gąsior et al., 2015).

More and more clinical and methodological papers highlight that respiration should be taken into account, or at least monitored, in HRV studies (Brown et al., 1993; Williams and Lopes, 2002; Eckberg, 2003; Song and Lehrer, 2003; Yildiz and Ider, 2006; Beda et al., 2014; Heathers, 2014; Lehrer and Gevirtz, 2014; Quintana and Heathers, 2014; Quintana et al., 2016a; Laborde et al., 2017). In population with known fast breathing rate, like children, respiratory depth, and frequency are particularly associated with heart rate and its fluctuations and may influence HRV data independently of cardiac autonomic activity (Eckberg, 2009; Quintana et al., 2016b). Also in our study, we observed significant correlation between children's respiratory rate and HR, nevertheless, the correlation between HRV and HR was stronger than the association between HRV and breathing rate, and the statistical analysis revealed that respiratory rate was redundant in the multiple regression analysis. In our recent study, we have shown that the influence of breathing rate on HRV appears to be, at least in part, HR dependent, i.e., after HRV correction for prevailing HR, the correlation between breathing rate and HRV decreases (Gąsior et al., 2016).

In the present study, we calculated VLF power from short-term ECG recordings (i.e., lasting 5 min) which may be problematic from methodological point of view (Task Force, 1996; Heathers, 2014). However, this parameter was also considered in the previous study concerning reference values for short-term (5 min recordings) standard HRV in children

(Michels et al., 2013). Shaffer and Ginsberg in an overview paper concerning HRV metrics and norms state that "the VLF band requires a recording period of at least 5 min" (Shaffer and Ginsberg, 2017). Moreover, in our previous studies addressing large populations of patients after myocardial infarction, VLF parameter calculated from 512 R-R intervals turned out to be the strongest predictor of different causes of death (Sacha et al., 2013a, 2014). Therefore, we included this parameter in the present analysis despite the short-term nature of ECG recordings. This may enable future studies aiming to explore its clinical value in pediatric populations with various disorders and consequently may help to understand the nature of this parameter.

To make our data pragmatic and comparable for other studies: (i) we calculated short-term time- and frequency-domain HRV parameters by using actual version of a very popular and free tool: Kubios HRV Standard ver. 3.0.2 (Tarvainen et al., 2014, 2017); (ii) in spectral analysis, we used frequency bands up to 0,5 Hz which is appropriate for children populations; (iii) we presented frequency-domain HRV parameters calculated with both fast Fourier transform and autoregressive method; and (iv) we provide the Excel sheet calculator to facilitate computation and assessment of corrected HRV parameters.

Limitations

A limitation of this study is the relatively narrow age range for which these normal limits can be applied, i.e., 6–13 years. However, this age period corresponds to the considerable developmental changes in both circulatory and neurological systems, and therefore the HRV normative values for this life period may be very useful in different clinical scenarios.

Finally, the norms can only be applied in Caucasian children. Caution is needed in applying the normative HRV values for other populations as the significant ethnic differences of HRV in children and youth were shown (Wang et al., 2005; Reed et al., 2006; Eyre et al., 2013). Future studies using approach considering differences in average HR may help to identify ethnic differences in corrected HRV indices.

CONCLUSION

This study provides HRV normative values for school-aged children which have been developed independently of their major determinants, especially related to average HR. An Excel sheet calculator is provided to increase accessibility (**Supplementary Table S11**, Sheet "Corrected HRV Calculator") and simplifies determination of HRV parameters from an individual child and if values are within normal limits.

AUTHOR CONTRIBUTIONS

JG, JS, and MP conceived and designed the experiment. JG and MP acquired the data. AT, TK, and BW analyzed the data for ECG assessment. JZ analyzed the data for respiratory rate calculations. JG and JS analyzed the data for HRV and statistical analysis. JG, JS, MP, JZ, PJ, AT, TK, BW, and MD interpreted the data. JG, JS, MP, JZ, PJ, AT, TK, BW, and MD drafted the work and revised it critically for important intellectual content. JG, JS, MP, JZ, PJ, AT, TK, BW, and MD approved the final version of the manuscript to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01495/full#supplementary-material

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Measures of Heart Rate Variability in 24-h ECGs Depend on Age but Not Gender of Healthy Children

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Many methods computing heart rate variability (HRV) have been applied in studies in children. Not all of these methods have a comprehensive physiological interpretation, and not all of studies are in agreement with the Task Force Standards on HRV from 1996, and the New Joint Position Statement on the advances of HRV from 2015. The study aim was to analyse HRV in the 24-h ECGs of healthy children by the Poincare plots and Lomb-Scargle periodograms, and to follow proper HRV recommendations. Additionally, we investigated the associations between age, children's sex and measured HRV indices. One hundred healthy children, aged 3–18 underwent 24-h ECG Holter monitoring. HRV was analyzed by the Poincaré plots and spectral by Lomb-Scargle periodograms of RR intervals. The Mann-Whitney test was used to compare sex differences in HRV, the van Elteren's test was used to correct for the age-gender interaction, and non-parametric Spearman correlation was applied to analyse the association between age and HRV indices. None of the HRV measures differed significantly between boys and girls. None of the HRV indices was modified by the age-gender interaction. There were statistically significant associations of age with measures of ultra-low (rho = 0.42; $p < 0.0001$), very low (rho = 0.35; $p = 00004$) and low (rho = 0.30; $p = 0.0028$) frequency powers, the ratio of the low to high frequency power (rho=0.38; $p = 0.0001$), indices of long-term (SD2; rho = 0.37; $p = 0.0002$) and total (SDNN; rho = 0.33; $p = 0.0008$) HRV, and the contribution of the long-term HRV to total HRV (CL; rho = 0.32; $p = 0.0012$). In general, HRV parameters derived from the analyses of Poincaré plots and Lomb-Scargle periodograms appear not to be affected by gender, however, most of them increase with age in the 24-h ECG recordings in healthy children.

Keywords: heart rate variability, healthy children, poincare plots, lomb-scargle periodograms, short-term HRV, long-term HRV, holter electrocardiograms

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INTRODUCTION

Heart rate variability (HRV) is the physiological variation in the duration of cardiac cycles. HRV is analyzed by many different mathematical algorithms (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015). For many years HRV has been calculated with the use of stand-alone computers, nowadays it can easily

be computed by mobile and wearable devices (Heathers, 2013; Guzik and Malik, 2016). So far HRV analysis has been applied in research, for both clinical and non-clinical purposes (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015). Some examples of HRV applications are prediction of the risk of premature mortality after myocardial infarction (Bigger et al., 1992; La Rovere et al., 1998; Stein and Reddy, 2005; Guzik et al., 2012) or development of congestive heart failure (Patel et al., 2017), diagnosis of autonomic dysfunction in diabetes (Akinci et al., 1993; Schein et al., 2009), non-invasive estimation of the autonomic modulation of the cardiovascular system during stress (Srivastana, 2014), relaxation (Quintana and Heathers, 2014) or the assessment of the effects of physical training on fitness level (Bernardi et al., 2001; Makivić et al., 2013; Sanchez-Gonzalez et al., 2015). All of these are the reasons why the interest in HRV is growing both in clinical and physiological studies.

There are many mathematical methods applied to compute HRV—they may be grouped into statistical, spectral, graphical, non-linear, complexity, or information based (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015). Among all of the methods and parameters which are in use only some are really comprehensive, have a good physiological interpretation or are perhaps better suited for the analysis of cardiovascular time series of RR intervals. The most classic parameters such as the mean duration of the RR interval or standard deviation of NN intervals (normal-to-normal RR intervals) (SDNN) are examples of HRV measures which are widely understood (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015). Some other parameters like SD1 and SD2 (see Methods for their explanation) from the Poincare plot analysis of RR intervals, have excellent physiological explanation (Brennan et al., 2001; Guzik et al., 2010; Sassi et al., 2015). SD1 depends on instant changes of each pair of heart beats, i.e., two consecutive RR intervals, and thus describes the shortest possible HRV (its physiological interpretation is identical with rMSSD, i.e., the root mean square of the successive differences between RR intervals, and in fact both are rescaled by a constant, i.e., the square root of 2) (Brennan et al., 2001; Guzik et al., 2007, 2010; Sanchez-Gonzalez et al., 2015; Sassi et al., 2015). On the other hand, SD2 depends on the changes of the mean duration of each pair of heart beats, alters much more slowly and thus is considered as an index of the long-term HRV (Brennan et al., 2001; Guzik et al., 2010; Sassi et al., 2015). Finally, there are several methods applied for the spectral analysis of HRV, for example: Discrete Fourier Transform by Fast Fourier Transformation, autoregressive models, discrete wavelet transform or Lomb-Scargle periodograms all of which are believed to retrieve information from the autonomic regulation of heart rate by reflecting vagal tone and sympatho-vagal balance (Lomb, 1976; Bigger et al., 1992; Moody, 1993; Parati et al., 1995; Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Eckberg, 1997; Bernardi et al., 2001; Verlinde et al., 2001; Pichon et al., 2006; Rajendra Acharya et al., 2006; Piskorski et al., 2010; Cysarz et al., 2011; Heathers, 2014; Sassi et al., 2015). Whereas the most commonly applied Fast Fourier Transformation requires resampling of the RR interval time series to get all the samples evenly distributed, the Lomb-Scargle periodogram does not need this. The Lomb-Scargle method can be reliably and directly applied to the irregularly sampled data (Lomb, 1976; Moody, 1993; Laguna et al., 1998; Piskorski et al., 2010). According to Moody (1993), only the Lomb-Scargle method, compared with Fast Fourier Transform and autoregressive models spectra, produces robust power spectrum density estimates in the presence of irregularly sampled signal such as an instantaneous heart rate, which can be additionally entwined from time to time by artifacts or ectopic beats. This method avoids all mathematical and technical problems of resampling and replacement of outliers, and introduces few drawbacks of its own. Finally, Moody recommends the Lomb-Scargle method as a method of choice for the spectral analysis of HRV (Moody, 1993). On the other hand it could be argued that, in general, being a least squares fit to a model, Lomb-Scargle periodogram strongly reflects the assumptions of the model. Additionally, in this method the concept of phase is lost as well as the ability to study the effect of filtering on the signal. However, according to Laguna et al. (1998) these drawbacks are not limiting in the case of the RR intervals time series and HRV.

HRV has been studied for clinical and physiological purposes in children for many years, and the relationship of HRV with gender and age is one of such examples. Despite there being many studies on this topic, data on the effects of gender and age on HRV in children are sparse (Korkushko et al., 1991; Clairambault et al., 1992; Finley and Nugent, 1995; Goto et al., 1997; Umetani et al., 1998; Silvetti et al., 2001; Rękawek et al., 2003; Lenard et al., 2004; Fukuba et al., 2009; Cysarz et al., 2011; Michels et al., 2013; Seppälä et al., 2014; Gasior et al., 2015; Jarrin et al., 2015; Sharma et al., 2015). There are many technical and methodological limitations of most of the studies: using ECG or non-ECG signals (e.g., from chest heart rate monitors); whether identification of the beat types and thus potential inclusion of non-sinus beats was present; whether replacing interpolation of the missing RR intervals was performed; different sampling frequency of ECG from 100 to 1,000 Hz or higher; recording the signals at various conditions, for different length of time from 1 min to 24 h, with averaging the 1-h segments separately for day and night or no averaging for the whole 24-h recording; using very small samples of studied children; enrolment of children in either a narrow range of or even exact age, e.g., 10 years old, or kids in a very wide range of age, sometimes with addition of young adults up to 22 years old; using different criteria for the healthy status; applying different mathematical algorithms for the same HRV parameters; using either RR intervals or heart rate; normalizing RR intervals to heart rate, etc. As a result—in spite of a huge number of publications there are many disparities and no uniform conclusions can be made which complicates and limits the practical use of HRV among pediatricians. Whereas some studies report on the existence of sex differences in HRV parameters, some others deny it. Similar conflicting data are on the relation of HRV with age—whereas some reports show age dependence, others either do not analyse this issue at all or do not confirm its existence.

As already mentioned, both the Poincare plots and the analysis of Lomb-Scargle periodograms of RR intervals have good physiological interpretations, and using their algorithms for the irregularly sampled RR intervals does not break any mathematical assumptions and does not introduce any intermediate, artificial signals. Both methods can be applied to the 24-h ECG recordings. However, to the best of our knowledge none of these methods have been applied to the 24-h ECGs collected in healthy children. For this reason, the primary, practical aim of this study was to perform the analysis of HRV in the 24-h ECGs by the Poincare plots and Lomb-Scargle periodograms in healthy children, and our intention was to exactly follow the requirements of the Task Force Standards on HRV measurement published in 1996 and the new joint position statement on the advances of HRV from 2015 (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015, respectively). Therefore, the secondary aim of our study was to analyse the association of sex and age in healthy children with the applied measures of HRV in 24-h ECG Holter recordings.

MATERIALS AND METHODS

We selected 100 healthy children aged 3–18 years from 1.6 thousand patients visiting our Outpatient Cardiac Paediatric Clinic in the years 2011-2014. The typical reasons for visits of these children were the differential diagnosis of heart murmurs, fainting, palpitations, chest pain and impaired exercise tolerance. The primary selection criterion was the availability of information from a complete cardiac clinical assessment based on the medical history, physical examination, standard biochemical analyses, 12-lead resting ECG, 24-h Holter monitoring, and echocardiography. From these patients we only included children with no acute or chronic disease, not taking any medication, with no abnormalities in the physical examination with the exception of the presence of an innocent (revealed in the course of diagnostic approach) heart murmur, normal resting 12-lead ECG (normal PQ, QRS and QT duration, no signs of any cardiac chamber hypertrophy, dilation or strain, no ECG signs of any typical channelopathy), no serious ventricular and supraventricular arrhythmias (i.e., no tachycardias), no pathological bradycardia due to sinus node or atrioventricular node dysfunction in 24-h ECG Holter monitoring, no structural or functional abnormality in the resting transthoracic echocardiography, and with normal results of standard biochemical analyses. Standard 12-lead ECG was recorded in supine position at rest, and all measurements of PQ, QRS, and QT were done manually. For the calculation of the corrected QT (QTc) we used the Bazett's formula (Bazett, 1920) Additionally, we did not include children who suffered from any serious infection at least 4 weeks before visiting the Outpatient Clinic as well as excluding all subjects participating in endurance sports. Consequently, all children accepted for this study were healthy.

Parents of all children as well as children of at least 7 years old themselves gave their informed consent for the enrolment to the study. We received permission from the local University Bioethical Committee.

METHODS

24-h ECG Holter Recording

Children underwent an ambulatory 24-h three-channel ECG Holter recording (Schiller Medilog Darwin, Schiller, Switzerland and since 2014 Schiller Medilog Darwin 2, Schiller, Switzerland) with 1,000 Hz sampling frequency of ECG. Initially, all recordings were automatically analyzed, then visually scanned and inspected to determine whether all beats were classified appropriately. If necessary, all misclassified beats and artifacts were manually corrected. The analyzed recordings were then exported to text files containing the duration of each cardiac cycle and an annotation of the beat type (sinus, supraventricular, ventricular or a technical artifact).

Heart Rate and Heart Rate Variability

According to recommendations on HRV measurement (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996), only RR intervals of sinus origin were used for the quantification of the mean RR interval and HRV parameters. The following HRV parameters were quantified (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Brennan et al., 2001; Guzik et al., 2010; Piskorski and Guzik, 2011; Sassi et al., 2015):

- SDNN—standard deviation of normal-to-normal RR intervals as a measure of total HRV;
- SD1—standard deviation measuring the dispersion of points in the Poincare plot of RR intervals across the identity line. It is a measure of short-term HRV arising from instant beat-to-beat changes in the duration of RR intervals (SD1 is a rescaled root mean square of the successive differences—rMSSD—of RR intervals with identical physiological interpretation). Importantly, the Poincare plot analysis used for the quantification of HRV parameters presented here is defined in the $RR_n - RR_{n+1}$ space—meaning that each point in the Poincare plot is described by the two neighboring RR intervals: the current RR interval (RR_n) and the next RR interval (RR_{n+1});
- SD2—standard deviation measuring the dispersion of points in the Poincare plots of RR intervals along the identity line as a measure of the long-term HRV arising from the changes of the mean of two consecutive RR intervals;
- CL—the contribution of the long-term HRV to the total HRV that is a percentage of the long-term variance ($SD2^2$) of RR intervals to doubled total variance of RR intervals ($SDNN^2$).

$$CL = (SD2^2)/(2 \times SDNN^2)[\%].$$

The doubled total variance of RR intervals is a sum of the short-term variance ($SD1^2$) and the long-term variance ($SD2^2$):

$$SD1^2 + SD2^2 = 2 \times SDNN^2,$$

Therefore, the contribution of the long-term variance of RR intervals, i.e., CL, to the total HRV is always opposite to the short-term (CS) contribution to the total HRV, and both sum up to 100%:

$$\begin{aligned} CS &= (SD1^2)/(2 \times SDNN^2)[\%] \\ CS + CL &= 100\% \\ CL &= 100\% - CS \end{aligned}$$

To avoid showing just mathematical rather than physiological associations, we only present the results for CL, without CS. The interpretation of CL is how much total HRV comes from the long-term variability of RR intervals, and that the remaining part origins from CS (i.e., short-term HRV). Because this is a relative measure with total HRV represented by 100%, the CL is self-explanatory—it helps to understand that as CL cannot reach the value of either 0 or 100% it is instantly obvious that the remaining part of total HRV must come from the short-term HRV.

For the spectral analysis the method of Lomb-Scargle periodograms was applied (Lomb, 1976; Moody, 1993; Laguna et al., 1998; Piskorski et al., 2010), and the following parameters were calculated (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015):

- ULF—the power of ultra-low frequency (0.00–0.0033 Hz) of RR intervals;
- VLF—the power of very low frequency (0.0033–0.04 Hz) of RR intervals;
- LF—the power of low frequency (0.04–0.15 Hz) of RR intervals;
- HF—the power of high frequency (0.15–0.4 Hz) of RR intervals;
- LF/HF—the ratio of the powers of LF to HF;

We have deliberately omitted the HF and LF in normalized units since there is a direct mathematical relationship between the two (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Eckberg, 1997; Burr, 2007; Piskorski et al., 2010; Heathers, 2014; Sanchez-Gonzalez et al., 2015; Sassi et al., 2015). Therefore, information redundancy is inherent in the normalized spectral HRV measures with respect to each other ($LF_{nu} = 1 - HF_{nu}$) and also with respect to the LF/HF ratio ($LF/HF = LF_{nu}/(1 - LF_{nu})$) (Burr, 2007; Piskorski et al., 2010; Heathers, 2014; Sanchez-Gonzalez et al., 2015). Therefore, the physiological information derived from LF/HF, LF_{nu}, and HF_{nu} is the same. We did not focus on the total power of RR intervals from spectral analysis either, although it was calculated together with LF and HF with the Lomb-Scargle periodograms. The total spectral power of RR intervals is always an approximation of total variability and it is never more accurate than SDNN. Therefore, to avoid another redundancy, we used SDNN as the only measure of total HRV.

In-house software written in Python (Python Foundation, Ipswich, MA, USA) was applied for all analyses of RR intervals.

Statistical Analysis

The Shapiro-Wilk test showed that data distribution was non-Gaussian, and hence they were described as mean, standard deviation (SD), median and the 25th and 75th percentile. The comparison of HRV parameters between girls and boys was made with the non-parametric Mann-Whitney test. To address the issue of potential interaction between age and children's sex in the Man-Whitney test we controlled for the age factor by carrying out the stratified van Elteren's test, which is an extension of the Man-Whitney test (Kawaguchi and Koch, 2010, 2015). All children were divided into three age groups, i.e., 3–6 years old corresponding to early childhood (Group 1); 7–12 years old corresponding to preadolescence (Group 2); and 13–18 years old, i.e., adolescence (Group 3). This division also corresponds to the school system in Poland according to the children's age, i.e., preschool period for children <7 years old; primary school for children between 7 and 12 years old, and the middle and high school for children >12 years old. The comparison between age groups was made with the use of the non-parametric Kruskal-Wallis test with post-tests. Since the age group is an ordered qualitative factor, the Jonckheere-Terpstra trend test was applied to evaluate the hypothesis that the medians of the analyzed parameters are ordered either in an increasing or decreasing direction according to the order of the qualitative factor (Bewick et al., 2004; Sheskin, 2011). Additionally, for the analysis of the association between age and HRV parameters the non-parametric Spearman correlation was applied. Statistical analyses were carried out with Prism for Windows (GraphPad, USA) and Medcalc for Windows (Medcalc, Belgium). Only $p < 0.05$ were considered significant.

RESULTS

Clinical Characteristics and Results of HRV Analysis According to Gender

There were 51 girls and 49 boys—their clinical characteristics and the results of the comparison by the Mann-Whitney test are shown in **Table 1**. Girls were significantly older (two-years difference in median age), had shorter duration of QRS (10 ms difference in median duration of QRS) and longer duration of QTc (7 ms difference in median duration of QTc) than boys. No other clinical parameters, including all HRV indices, were different between girls and boys.

Clinical Characteristics and Results of HRV Analysis According to the Interaction of Gender and Age

The Elteren's test revealed that the interaction between age and gender of the studied children might have some effect on the duration of QRS, QTc, and mean RR interval. None of the HRV parameters was influenced by the interaction between children's sex and age.

TABLE 1 | Clinical characteristics and results of HRV analysis as well as comparison between girls and boys by the Mann-Whitney test, and the van Elteren's test for the analysis of the influence of the interaction between age and sex on the analyzed parameters.

	Girls $N = 51$					Boys $N = 49$					M-W test p-value	van Elteren's test
	Mean	*SD*	Median	25th p.	75th p.	Mean	*SD*	Median	25th p.	75th p.		
Age [years]	13.9	3.7	15.0	13.0	16.0	12.3	3.9	13.0	9.8	15.0	0.0025	–
PQ [ms]	129.0	14.7	130.0	120.0	140.0	129.9	17.8	130.0	120.0	142.8	0.6125	0.5654
QRS [ms]	83.8	8.6	80.0	80.0	90.0	87.2	9.0	90.0	80.0	91.0	0.0422	0.0001
QTc [ms]	398.1	16.7	397.0	387.3	411.5	387.6	21.7	390.0	370.8	400.0	0.0288	0.01394
Supraventricular beats in 24-h ECG [%]	0.000235	0.0009293	0.0	0.0	0.0	0.00173	0.006480	0.0	0.0	0.0	0.3575	0.05593
Ventricular beats in 24-h ECG [%]	0.000176	0.0005179	0.0	0.0	0.0	0.205	1.4248	0.0	0.0	0.0	0.9589	0.1525
Duration of ECG recording [min]	1287.157	119.5533	1297.832	1202.0	1385.2	1313.218	81.7335	1302.376	1259.1	1370.6	0.5372	0.6075
Mean RR [ms]	736.2	73.0	736.5	688.3	783.1	744.9	87.2	758.6	679.2	805.6	0.8930	0.0242
SDNN [ms]	159.0	43.1	161.5	132.6	190.2	158.0	39.9	157.1	126.1	190.3	0.4139	0.5090
ULF [ms^2]	21732.3	12472.1	19582.6	12536.1	30669.4	19821.3	10731.4	18062.3	10912.7	26070.9	0.2262	0.8763
VLF [ms^2]	3501.0	1648.8	3396.7	2095.2	4541.7	3928.5	2241.4	3279.0	2256.6	5845.1	0.8442	0.1359
LF [ms^2]	1925.6	1164.9	1765.5	1072.6	2399.0	2097.8	1107.5	1723.1	1319.4	2906.1	0.8066	0.0813
HF [ms^2]	1844.4	2077.5	1476.4	599.4	2139.8	1957.5	1734.5	1376.0	760.8	2286.6	0.3466	0.4395
LF/HF []	1.5	0.7	1.4	1.1	1.8	1.5	0.7	1.3	0.9	2.1	0.2236	0.7865
SD1 [ms]	47.7	25.6	43.9	28.5	56.5	50.3	23.6	48.2	32.3	60.0	0.6766	0.4474
SD2 [ms]	218.3	58.5	223.7	179.3	263.7	216.1	54.5	210.7	172.2	261.7	0.7433	0.5152
CL [%]	95.1	4.3	96.5	94.2	97.6	94.4	4.4	95.9	93.3	97.2	0.1030	0.5354

Clinical Characteristics and Results of HRV Analysis in Relation to Age

There were 7 children in the first age group (3 girls), 28 in the second (9 girls), and 65 in the third (39 girls). A summary of clinical characteristics, results of HRV analyses and statistical comparisons by the Kruskal-Wallis test are shown in **Table 2**. The results of the *post-hoc* analysis with trend analysis between age groups by the Jonckheere-Terpstra trend test are shown in **Table 3** (only for parameters with a significant *p*-value in the Kruskal-Wallis test).

The variance of the duration of PQ and QTc was not related to the age group. However, QRS duration significantly increased with age, and the trend for this increase was statistically significant. Although there was no significant relationship between the average duration of ECG Holter recording along age groups, the number of beats rejected from analysis was higher in older children than in the youngest. Nevertheless, there was no significant trend in the number of rejected beats with increasing age.

For HRV, with the exception of HF power and SD1, all other HRV parameters were significantly different between age groups. The duration of mean RR and values of SDNN, ULF, VLF, LF, LF/HF, and SD2 significantly increased while the relative contribution of long-term HRV to total HRV (CL) significantly decreased across age groups.

Figures 1, **2** show scattergrams for the relations between analyzed HRV parameters and children's age. The results of non-parametric correlation analysis between the age of healthy children and the studied HRV parameters are shown in **Table 4**. There was no significant correlation between the age of children and the power of HF or value of SD1. There was a significant and positive correlation between the age and the duration of mean RR interval, SDNN, ULF, VLF, LF, LF/HF, SD2, and CL. Out of all significant correlations, the strongest correlation was between the age and mean RR interval, and ULF; however, the strength of these correlations was at most moderate. The remaining significant correlations were rather weak. As shown in **Figures 1** and **2**, the analyzed relations between HRV and age appear to be non-linear.

DISCUSSION

With this study we applied the Poincare plot analysis and the Lomb-Scargle periodograms to the RR interval time series derived from the 24-h ECGs. The application of both methods for HRV to the 24-h ECGs in healthy children is one example of novelty brought by our study. Another novelty is the use of an index CL derived from the Poincare plots analysis of RR intervals which helps to estimate what is the relative contribution of the long-term variability to the total HRV at the cost of the short-term variability. Additionally, we provide reference values for these methods and a set of parameters derived from them—this is important as we carefully evaluated the health status of the enrolled children, used the recommended approach to HRV analysis (detailed ECG analysis with identification of beat types and using RR intervals only of sinus origin, high sampling rate of

TABLE 2 | Clinical characteristics and results of HRV analysis of studied healthy children in three age groups with results of the Kruskal-Wallis test.

	1st age group 3-6 years old *N* = 7					2nd age group 7–12 years old N = 28					3rd age group 13–18 years old N = 65					K-W test *p*-value
	Mean	***SD***	**Median**	**25th p.**	**75th p.**	**Mean**	***SD***	**Median**	**25th p.**	**75th p.**	**Mean**	***SD***	**Median**	**25**	**75 P**	
Age [years]	4.4	1.3	4.0	3.3	5.8	9.6	1.7	10.0	8.0	11.0	15.5	1.6	15.0	14.8	17.0	
PQ [ms]	118.9	8.7	120.0	110.5	127.5	127.9	18.0	128.0	114.0	140.0	131.2	15.7	130.0	120.0	140.0	0.1120
QRS [ms]	77.0	13.0	80.0	65.5	87.5	83.3	8.1	81.0	79.0	90.0	87.3	8.1	86.0	80.0	90.0	0.0270
QTc [ms]	388.7	22.2	388.0	373.8	395.0	391.8	16.4	393.0	381.0	401.5	393.9	21.2	395.0	379.5	410.5	0.6176
Supraventricular beats in 24-h ECG [%]	0	0	0	0	0	0.8	2.8	0	0	0	1.1	5.4	0	0	0	0.5739
Ventricular beats in 24-h ECG [%]	0.4	1.1	0	0	0	0.2	1.0	0	0	0	1.5	4.4	0	0	0	0.5274
Duration of ECG recording [min]	1312.3	44.1	1327.5	1277.2	1345.6	1311	101.6	1306.1	1235.4	1419.2	1293.8	108.6	1293.8	1223.2	1380.3	0.8001
Mean RR [ms]	643.2	40.1	656.7	621.0	668.8	701.1	73.2	705.8	637.1	756.8	767.9	70.8	770.1	714.1	812.8	0.0000
SDNN [ms]	125.5	34.3	126.3	93.7	147.8	148.0	42.4	139.3	118.0	177.9	166.6	39.3	165.6	139.6	192.6	0.0111
ULF [ms^2]	11638.2	7240.5	7617.0	6955.2	16066.3	16741.7	10074.6	13420.9	9735.0	21376.7	23528.6	11732.1	23091.7	14798.3	31390.6	0.0006
VLF [ms^2]	1982.0	1172.1	1891.0	1102.1	2739.2	3169.6	1730.7	2475.7	1870.1	4152.9	4129.6	1984.3	3919.1	2787.9	5191.7	0.0025
LF [ms^2]	1171.9	682.4	1213.8	717.6	1358.3	1809.3	1022.8	1558.6	1048.6	2444.5	2186.7	1174.9	1977.6	1338.6	2839.3	0.0304
HF [ms^2]	1646.0	1462.8	1537.1	548.0	2252.4	2258.1	2563.8	1372.1	734.8	2726.5	1772.9	1606.2	1476.4	676.7	2058.0	0.8695
LF/HF [ ]	1.0	0.6	0.8	0.6	1.1	1.2	0.6	1.1	0.9	1.5	1.6	0.7	1.6	1.2	2.2	0.0031
SD1 [ms]	51.7	27.4	59.5	25.4	67.2	51.0	29.1	45.1	28.6	66.2	47.8	22.4	44.5	32.3	55.9	0.9314
SD2 [ms]	168.7	44.6	168.4	129.3	195.5	201.3	55.5	191.0	162.3	238.9	229.2	53.8	230.4	190.7	267.5	0.0039
CL [%]	91.4	6.7	92.8	89.4	96.3	94.0	4.6	95.5	92.4	97.2	95.5	3.7	96.6	94.6	97.7	0.0228

TABLE 3 | Results of the *post-hoc* comparison of clinical characteristics and results of HRV analysis between age groups (only $p < 0.05$), and the Jonckheere-Terpstra trend test for the trend analysis along age groups.

	1st vs. 2nd age group	1st vs. 3rd age group	2nd vs. 3rd age group	J-T trend test
Age	$p < 0.05$	$p < 0.05$	$p < 0.05$	<0.00001
QRS		$p < 0.05$	$p < 0.05$	0.00836
Mean RR	$p < 0.05$	$p < 0.05$	$p < 0.05$	<0.00001
SDNN		$p < 0.05$	$p < 0.05$	0.00340
ULF		$p < 0.05$	$p < 0.05$	0.00010
VLF		$p < 0.05$	$p < 0.05$	0.00090
LF		$p < 0.05$		0.01693
LF/HF		$p < 0.05$	$p < 0.05$	0.00073
SD2		p <0.05	$p < 0.05$	0.00105
CL		p <0.05		0.00721

the ECG signal, and 24-h duration of these recordings). We have also found that the sex of healthy children does not contribute to the applied HRV indices. For the age, however, we found a statistically significant relationship with the values of most measured HRV parameters. It appears that as a child advances in age in the range of 3–18 years, most HRV measures (ULF, VLF, LF, LF/HF, SDNN, SD2, CL) increase. The only HRV indices which do not alter significantly with age are those reflecting fast-oscillations (HF) or short-term (SD1) variability of RR intervals, both of which correspond to HRV phenomena on short time scales.

Clinical Studies on HRV in Children

There is a substantial number of reports on the clinical use of HRV in children, including newborn babies and infants. Reduced HRV, i.e., total power, VLF, LF, and HF, was observed in a group of 112 children with Fontan circulation compared with 66 control subjects (Dahlqvist et al., 2012). Massin and von Bernuth (1998) found reduced HRV in children with congenital heart disease, and that HRV was lower in patients with more advanced heart failure assessed by the NYHA functional class. Dias de De Carvalho et al. (2014) observed that children with the Attention Deficit Hyperactivity Disorder (ADHD) had increased HRV indices describing the parasympathetic activity (i.e., HF, rMSSD or percentage of differences between adjacent normal-to-normal intervals greater than 50 ms—pNN50) compared with control group. Kardelen et al. (2006) reported that HRV parameters were markedly reduced in children with type 1 diabetes compared with healthy children. Akinci et al. (1993) found using 24-h ECG Holter recordings that diabetic children with poor glycaemic control had significantly reduced HRV indices compared with healthy children (for example LF and HF). Soares-Miranda et al. (2011) noticed that HF is reduced in girls with central fat above the median value compared with girls with lower amount of central fat. Spassov et al. (1994) analyzed the influence of the retardation of intrauterine growth on heart rate and HRV, and reported that small-for-gestational age newborn babies had shorter RR interval and reduced HRV in comparison with the appropriate-for-gestational age newborns during sleep. Schechtman et al. (1989) found decreased HRV in the long-term recordings obtained from infants under 1 month who later succumb to sudden infant death syndrome (SIDS). However, Antila et al. (1990) did not observe any significant differences in HRV between 17 infants with SIDS and 23 healthy infants. These examples clearly show that there is a lot of interest in HRV in children in a variety of clinical conditions.

Physiological Studies on HRV in Children

Sex of Children and HRV

The issue of the children's sex and its relation to HRV was a subject of several studies. Silvetti et al. (2001) studied time domain HRV parameters in 24-h ECGs in 103 healthy children starting from one-year-old babies up to adolescents and found that neither SDNN, pNN50 nor rMSSD were related to gender. Rękawek et al. (2003) carefully investigated a group of 372 children aged from 4 to 18 years and measured HRV by many time domain and spectral HRV parameters in the 5- and 20-min ECG segments selected from the day and night of 24-h ECG Holter recordings. In their comments Rekawek et al. report that sex was the least significant contributor to time domain HRV and the most significant to the total power of HRV—unfortunately the authors do not present detailed data for this type of analysis, and by studying the tables with the summary of the results the reader can guess that the sex effects on HRV were either missing or very week. Seppälä et al. (2014) examined 465 mainly prepubertal children in a very narrow age range of 6–8 years. HRV was analyzed in the resting ECGs of 1- and 5-min duration, and the authors did not find any significant difference between girls and boys in the studied HRV parameters. Umetani et al. (1998) studied time domain HRV parameters in 24-h ECGs in a group 260 healthy subjects in a wide range of age between 10 and 99 years old. In this study, there were only 30 children up to 19 years old, however for the analysis of sex effects, these children were combined with adults up to 29 years old—in summary, there were significant differences for the time domain parameters such as SDNN, SDANN (standard deviation of 5-min means of RR intervals), rMSSD or pNN50 between combined groups containing 32 boys and young men vs. 40 girls and young women—no separate analysis of sex differences between the studied children were shown. Goto et al. (1997) investigated 25 healthy boys and 35 healthy girls between 3 and 15 years old and found no significant differences by sex in any of the studied HRV indices, neither in time nor in frequency domains. Michels et al. (2013) studied a group of 460 children in the age between 5 and 10 years old. The children were selected by random cluster sampling from the region of Aalter in Belgium, and RR intervals were derived not from an ECG but from the elastic electrode belt Polar Wearlink (Polar, Finland) placed around the chest. For the HRV, both time and frequency domain parameters were studied in 5-min segments of RR intervals. Mean RR interval, SDNN, rMSSD, pNN50, the powers of VLF, LF, and HF were significantly larger in boys than in girls, but there was no significant difference in any of the relative measures of spectral HRV indices (LF/HF, LFnu, HFnu). Fukuba et al. (2009) examined 48 boys between 8 and 14 years old and 88 girls between 8 and 20 years old who were asked to breathe periodically at a rate of 15 breaths/min. For

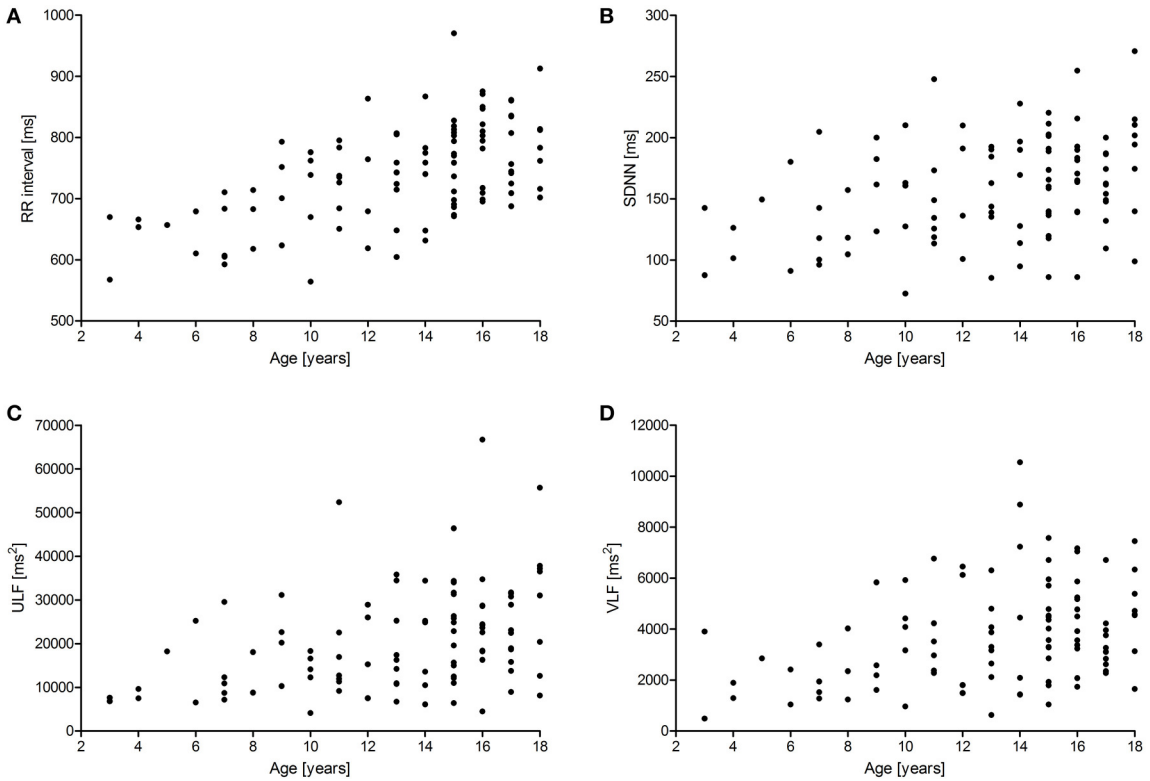

FIGURE 1 | Scattergrams showing relations between children's age and mean RR interval (A), SDNN **(B)**, the power of ULF **(C)**, and VLF **(D)**. For abbreviations, please refer to the main text.

the HRV analysis, the authors used 3-min ECGs and measured the rate of HF to TP as an index of parasympathetic, and LF/HF as an index of sympathetic control of heart rate—they found no sex differences in these indices. Sharma et al. (2015) conducted a study in a group of 250 boys and 189 girls in the age between 12 and 17 years old who were then divided into non-athletes and athletes according to the definition that an athlete had represented the school at state, national or international level athletic interscholastic sport event and was undergoing supervised physical training. The time and spectral domain HRV was computed in RR intervals gathered with the use of the Bioharness sensor placed on a chest strap (Zephyr, USA) in 5-min resting recordings. Among the non-athletes, girls had significantly higher values of rMSSD, NN50, TP, LF, and HF than boys, and there were no significant sex differences for SDNN and LF/HF (and logically LFnu and HFnu). However, among athlete adolescents, girls presented significantly higher SDNN than boys, and there were no other significant differences among other HRV indices between sexes. Jarrin et al. (2015) investigated a large group of 1,036 children (481 boys and 555 girls) in a very narrow age range 9–11 years (mean 10 years old). The authors found many statistically significant differences (p at least <0.01) suggesting that boys, compared to girls, have significantly higher values of time and spectral HRV parameters with the exception of LF/HF which appeared to be lower. However, the analysis of mean values of these parameters and their standard deviations show that the differences in the means were rather small, for example the comparison of boys vs. girls for SDNN shows 89.4 $\pm$ 26.1 vs. 84.4 $\pm$ 23.1 ms, and for LF 1,587.7 $\pm$ 1,040.1 vs. 1,411.2 $\pm$ 934.4 ms^2, respectively. The most curious comparison is revealed for LF/HF where it is 2.1 $\pm$ 0.8 for boys, and 2.2 $\pm$ 0.9 for girls. Galeev et al. (2002) examined probably the largest group of 5,400 children in the age between 6 and 16 years old for the purposes of HRV study, and finally selected 300 children for each year of age collecting together 3,300 sets of RR intervals. For the spectral HRV analysis, 128 consecutive RR intervals were selected whether for the time domain HRV 2000 consecutive RR intervals were chosen. SDNN was significantly higher in boys than in girls for 12, 13, and 14 years old children. TP was significantly higher in boys in the age interval 11–15 years old, VLF was larger in boys in the age 13–15 years but lower for 12 years old. Sex differences in LF and HF were present for children in the age between 15 years but in changing patterns, i.e., once these parameters were higher in boys, then in girls, and then again in boys. The LF/HF was larger in boys than in girls between 12 and 15 years old. For children at age of 16 years there were no sex differences in any of the HRV studied parameter. Such studies as Jarrin et al. (2015) and Galeev et al. (2002) raise a question whether all statistically significant differences are clinically relevant? Whether, in fact, the small differences in the values of HRV measures are statistically different because of the mass effect, i.e., large sample sizes influencing *p*-value

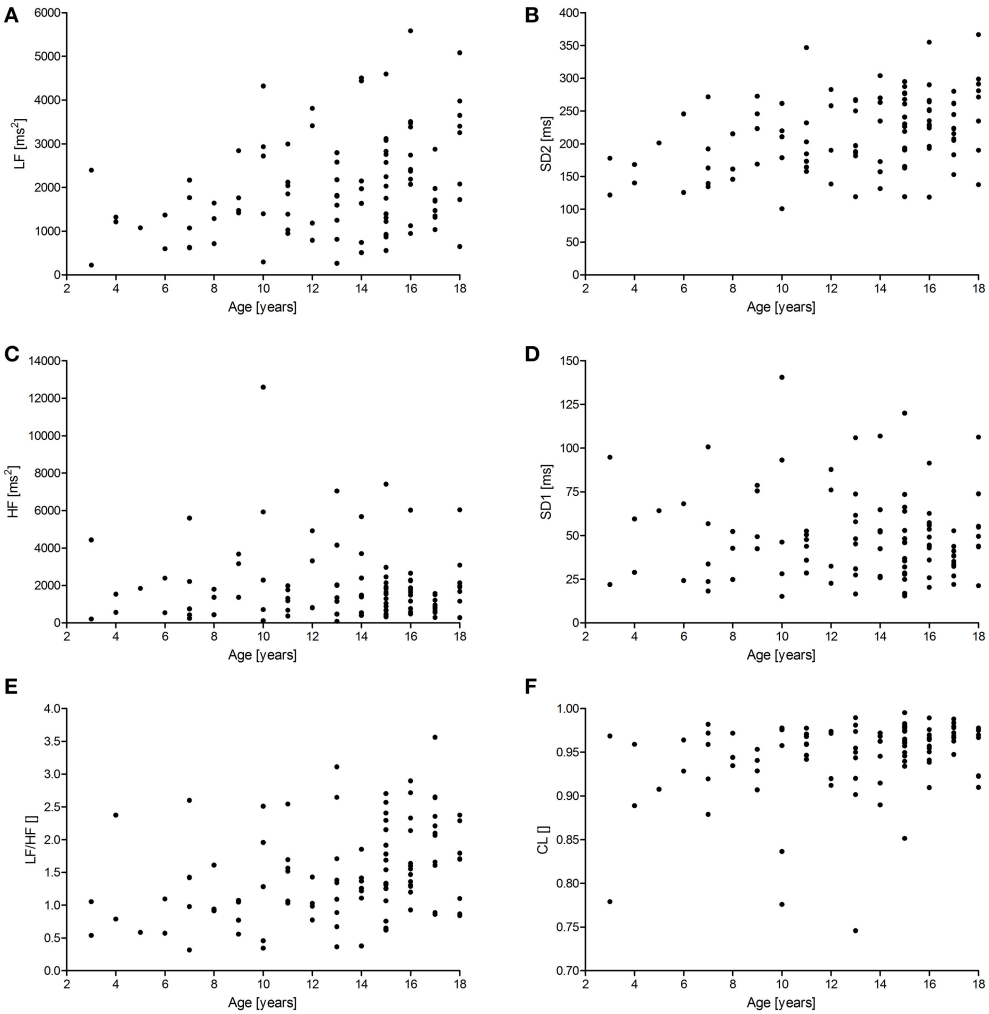

FIGURE 2 | Scattergrams showing relations between children's age and the power of LF (A) and HF **(C)**, SD2 **(B)**, SD1 **(D)**, LF/HF **(E)**, and CL **(F)**. For abbreviations, please refer to the main text.

(Farland et al., 2016). Gasior et al. (2015) recorded a resting ECG in 158 boys and 173 girls in age between 6 and 13 years old and selected 5-min segments for the time and spectral HRV analysis. During their analysis the authors manually corrected the erroneous beats by removing one RR interval before and one after each non-sinus beat, and then replacing the missing RR intervals by mathematically interpolated RR intervals. Compared with girls, boys presented significantly higher HRV values (SDNN, rMSSD, pNN50, TP, LF, and HF) but lower heart rate. However, in the linear multiple regression analysis adjusted for sex, all HRV parameters were significantly related to heart rate but not sex of the children—this suggests that if there are any sex differences in HRV in children they are rather an effect of heart rate than the sex itself. Cysarz et al. (2011) collected up to 24-h ECG Holter recordings in 409 healthy children, adolescents and young adults in the age range between 1 and 22 years. In this group there were 220 girls or women and 189 boys or men. In addition to a set of parameters assessing the non-linear measures for irregularity and fractal properties of RR intervals, they computed SDNN and ULF for the whole 24-h ECGs and, additionally, VLF, LF, HF, and LF/HF for each consecutive 1-h epoch of the recording. These 1-h epochs for VLF, LF, HF, and LF/HF were then averaged separately for daytime (from 9 a.m. to 6 p.m) and night-time (from 0 a.m. to 6 a.m.). Both SDNN and all spectral measures of HRV were significantly lower in girls and young women than in boys and young men. With our study we observed no differences in HRV parameters (neither from the spectral analysis by Lomb-Scargle method nor Poincare plots) calculated in the 24-h ECGs between healthy girls and boys. Additionally, we did not find any influence of the interaction between age and children's gender on the studied HRV parameters. In general, it may be concluded that in contrast to adults, gender seems to have no influence on HRV in children, at least with the methods we used and for the 24-h ECGs. In summary of the gender relation to HRV, the available data are sparse and contradicting. Some authors show that boys have significantly higher HRV, some other show the reverse effects whereas some, including us, reveal no association of HRV with the sex of children.

TABLE 4 | Results of Spearman correlation (rho co-efficient) between the age of healthy children and studied HRV parameters from the 24-h ECG Holter recordings.

	Rho	*P*-value
Mean RR	0.55	< 0.0001
SDNN	0.33	0.0008
ULF	0.42	< 0.0001
VLF	0.35	0.0004
LF	0.30	0.0028
HF	−0.02	0.8758
LF/HF	0.38	0.0001
SD1	−0.04	0.6992
SD2	0.37	0.0002
CL	0.32	0.0012

Age of Children and HRV

There are numbers of studies investigating the relationship between the age of healthy children and HRV. Clairambault et al. (1992) studied 24 healthy sleeping newborns who were born between 31 and 41 weeks of the conceptional age. They found that HRV measured by spectral analysis was increased from the premature (31–36 weeks) to the intermediary (37–38 weeks) for both HF and LF, and to the full term (39–41 weeks) of the conceptual age for the LF. Silvetti et al. (2001), who investigated time domain HRV, found that there was a decrease of mean RR interval and SDANN with an increasing age of children (between the age of one year and adolescence), but no relation was observed with other HRV parameters. Goto et al. (1997) examined 60 healthy children aged 3–15 years for HRV. Time domain HRV measures showed a significant increase in SDNN in the group 3–9 years old and a decline in children 9–15 years old. For the spectral HRV assessment, 10-min recordings were used—the authors showed that HF and LF increased in children 3–6 years old and then gradually declined in children who were 6–15 years old. The LF/HF ratio increased in the group of 3–6 year old and then no significant differences were observed in older children up to 15 years old. Rękawek et al. (2003) investigated healthy children between 4 and 18 years old and found a significant correlation between several time domain HRV parameters (e.g., SDNN or SDANN) and age. However, similar correlation with age was absent for both rMSSD and pNN50. For the spectral HRV for selected 20-min ECGs from the day and night only some of these parameters (e.g., VLF, LF, HF, and LF/HF) were significantly correlated with age. Michels et al. (2013) in their study of children between 5 and 10 years old found that age correlated with both time and frequency domain parameters in boys and girls. Fukuba et al. (2009) did not find any significant correlation between age and neither the parasympathetic (HF/TP) nor sympathetic (LF/HF) indices of the control of heart rate. Although Sharma et al. (2015) provided reference data for many HRV parameters for children in the age between 12 and 17 years old, they did not report any association between age and the studied parameters. Gasior et al. (2015) did not find any significant correlation between age (children in the range 6–13 years old) and HRV parameters derived from time or frequency domains. Lenard et al. (2004) analyzed time domain and spectral HRV in 10-min resting ECGs in 137 healthy children and young adults divided into 4 age groups, i.e., 7–10 years old, 11–14 years old, 15–18 years old, and 19–22 years old. They found that only SDNN, LF, and HF were significantly larger in the oldest children 15–18 years old compared to the middle group between 11 and 14 years old. Finley and Nugent (1995) investigated 61 healthy children and young adults from 1 month to 24 years old using 24-h Holter ECG recordings. From these recordings segments of 1-h duration for the awake state, quiet and active sleep were chosen for the computation of spectral HRV indices. The power of LF and LF/HF changed significantly with age during both quiet and active sleep whereas HF only changed during quiet sleep. The total power was not related to age irrespective of the sleep or awareness state in this study. Massin and von Bernuth (1998) recorded 24-h ECGs in 210 children aged between 3 days to 14 years old to analyse time and frequency domain HRV indices, including SDNN, SDANN, rMSSD, pNN50, VLF, LF, HF, and LF/HF. They noticed that age was significantly and positively correlated with all studied HRV parameters. Galeev et al. (2002) who examined altogether 3,300 sets of RR intervals from children found that practically all of time and spectral HRV indices changed more or less with age, usually in a waveform. Compared with younger children, TP, VLF, and LF started to differ from the age of 11 while SDNN, rMSSD, and HF from the age of 12. In their study, Cysarz et al. (2011) found age related variation in the SDNN, ULF, VLF, LF, and LF/HF—the value of these parameters was, in general, increasing with age. In contrast, there was no relation between HF and age of the studied children and young adults.

In our study we also observed a series of significant correlations between age and most time domain, spectral and Poincare plot HRV parameters computed in the 24-h ECGs. Although the magnitude of these associations was rather modest to medium, most values of the HRV parameters increased significantly with age in the range of 3–18 years. This is true for the measures of total (SDNN), ultra low (ULF), very low (VLF) and low frequency (LF) as well as long-term (SD2) variability—all of these parameters are different parts of the total variance (i.e., squared SDNN).

Our observations on the relation of age with at least some of the HRV measures, i.e., SDNN and LF, are in concordance with the studies by other authors (Finley and Nugent, 1995; Goto et al., 1997; Massin and von Bernuth, 1998; Galeev et al., 2002; Rękawek et al., 2003; Lenard et al., 2004; Cysarz et al., 2011; Michels et al., 2013). However, it appears that not all HRV indices that contribute to total HRV show such a significant relationship with age. In our case, neither HF nor SD1 alter significantly with age. This observation is partially similar to the study of Silvetti et al., Rekawek et al., Gasior et al., and Cysarz et al. Silvetti et al. (2001) who reported that two measures of short-term variability, i.e., pNN50 and rMSSD (which is the rescaled SD1 and thus identical in interpretation with it) were only partially related to age—these are correlated with age only for children up to 10 years old—later, this relationship is no longer significant. Rękawek et al. (2003) did not find such correlation with either

RMSSD or pNN50 in their group. In spectral analysis, there were significant correlations between age and HF both for the 20-min ECGs from day and night, however these correlations were very weak (r coefficient −0.13 and −0.11, respectively). In the study by Gasior et al. (2015) no significant correlation between children's age and neither rMSSD nor HF were observed. Cysarz et al. (2011) found that HF was constant in children up to 13 years old and then slightly declined but in general it was not correlated with age. Although both HF and rMSSD (or SD1) come from different methods, in general they describe similar physiological phenomena of short-term or high frequency HRV (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Brennan et al., 2001; Guzik et al., 2007, 2010; Heathers, 2014; Sanchez-Gonzalez et al., 2015; Sassi et al., 2015), and according to the previous reports and our findings, we may conclude that the association between age and the measures of fast oscillations influences on HRV are either weakly or not at all correlated with the age of healthy children, at least when measured in 24-h ECGs by Poincare plots or Lomb-Scargle periodograms.

There are several differences between our study and the other investigations on HRV in healthy children. In a variety of studies, ECGs of different duration were used. This duration ranged from 1 min (Seppälä et al., 2014), through 3 (Fukuba et al., 2009), 5 (Rękawek et al., 2003; Michels et al., 2013; Seppälä et al., 2014; Gasior et al., 2015; Sharma et al., 2015), 10 (Goto et al., 1997; Jarrin et al., 2015), 20 (Rękawek et al., 2003) min to 5 (Goto et al., 1997), 6 for the night-time and 9 for the daytime (Cysarz et al., 2011), and 24 h (Umetani et al., 1998; Silvetti et al., 2001; Cysarz et al., 2011; our study). Galeev et al. used an unusual approach by analysing RR intervals of exactly 128 consecutive RR intervals for spectral HRV or at least 2000 RR intervals for time domain HRV (Galeev et al., 2002). Usually many different computational methods (time domain, spectral analysis by Fast Fourier Transform or Lomb-Scargle periodograms, and Poincare plots of RR intervals) were applied for HRV analysis. We used ECG Holter recorders with a sampling frequency of 1,000 Hz whereas in older studies it was lower, usually 128 Hz or sometimes 500 Hz. Higher sampling frequency increases the accuracy of RR intervals and thus HRV analysis (Piskorski and Guzik, 2011). Similarly to Laguna et al. (1998), for spectral HRV analysis we applied the Lomb-Scargle periodograms of RR intervals while other studies used Fast Fourier Transform (FFT), autoregressive models or wavelet decomposition analysis (Moody, 1993; Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Verlinde et al., 2001; Pichon et al., 2006; Rajendra Acharya et al., 2006; Cysarz et al., 2011; Heathers, 2014). The FFT requires that the signal is sampled at equal intervals and since this condition is broken in RR intervals, the signal needs to be resampled. In contrast, the Lomb-Scargle periodograms of the RR intervals approach does not have similar requirements and limitations (Lomb, 1976; Moody, 1993; Piskorski et al., 2010). It can use unequally sampled signals such a time series of RR intervals which is naturally irregular. According to the recommendation of Moody (1993) and studies by Laguna et al. (1998), the Lomb-Scargle periodograms are the method of choice for the spectral HRV analysis as this method is better suited for this purpose than FFT. Laguna et al. (1998) goes even further after studying the effects of uneven sampling and the spectrum obtained by FFT and the Lomb-Scargle method, and suggest that the Lomb-Scargle approach is superior to FFT. Most of the times both methods for spectral HRV will give similar results, but in cases of some unforeseen violations of the assumptions of the methods, the Lomb-Scargle periodogram is closer to reality than FFT. We are, however, aware that the Lomb-Scargle periodogram analysis of RR intervals has also some limitations which we mentioned earlier. One of such limitations is the loss of the information about phase in the analyzed signal, however this information is uninterpretable in case of spectral analysis of RR intervals and thus not used at all.

To the best of our knowledge, this study is the first report on the application of the Lomb-Scargle periodograms method to 24-h ECG Holter recordings in healthy children, and for this reason these results can be used as reference values by other authors. It also appears that we are the first to use Poincare plot analysis of RR intervals for HRV derived from 24-h Holter ECG recordings in this group. In addition, we also used a new parameter—CL—quantifying the relative contribution of the long-term HRV to total HRV. This parameter helps to understand what proportion of total HRV derives from the long-term HRV—the remaining amount comes from the short-term HRV. Since there is a positive relationship between the age of children and CL, it follows that with the process of growing up there is an accumulation of long-term effects at the cost of the short-term input to the total HRV. This might be caused by respiratory sinus arrhythmia and its gradually weakening effects on the heart rate and HRV with advancing age of children, and that other physiological effects start having stronger influences on HRV. On the other hand, CL might also be interpreted as a relative index reflecting the expression of respiratory sinus arrhythmia on HRV.

Limitations of the Study

Some limitations of our study must be recognized. First, we used HRV, which is an indirect measure of the cardiac autonomic control. However, HRV is commonly applied for this purpose in a number of studies and even recommended for this purpose in specific guidelines (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996; Sassi et al., 2015)—one of the most beneficial features of this approach is a complete lack of invasiveness of HRV measurement. Second, we studied only 100 healthy children. The reason was that selection process was very detailed, careful and required a lot of effort to select such children from over 1,600 patients visiting the Outpatient Cardiac Paediatric Clinic who underwent a detailed clinical evaluation including echocardiography and 24-h ECGs recording. We took care of the comfort of all studied children as well as data quality. During this process we did not use special forms or clinical/research tools designed for specific purposes like the Growing and Changing Questionnaire for the evaluation of maturity, the Eurofit fitness test battery or maximal oxygen consumption (VO2max) for the physical fitness or total body impedance for the body composition analysis (Michels et al.,

2013; Jarrin et al., 2015; Sharma et al., 2015)—looking at the possible associations between HRV measures and many potential covariates collected in the enrolled children was not the aim of our research. To get the best possible results we also applied a specific method for spectral HRV analysis, which is the Lomb-Scargle periodogram and that is recommended for the HRV analysis (Moody, 1993). Third, we also provide some completely new data, e.g., from Poincare plots and Lomb-Scargle periodograms analysis or with a new HRV measure, i.e., CL.

CONCLUSIONS AND POTENTIAL IMPLICATIONS

Based on previous studies which differ in so many aspects with our study it is difficult to clearly draw general conclusions on the relation of the HRV derived from 24-h ECGs to children's age and sex. For these reasons, we may make conclusions based only on our findings.

We did not find any gender differences in many HRV parameters computed for the 24-h ECGs by the Poincare plots and Lomb-Scargle periodograms in healthy children. However, we found that there is a significant correlation between age and the expression of most of HRV parameters which represent total HRV or its contributors from the long-term or ultra-low, very low and low frequency oscillations. We could not observe similar associations between age and the HRV contributors derived from the short-term effects or high frequency oscillations. The effects of age on the tested HRV indices was not modified by children's sex.

The potential implication of our study is using its results as reference values for the Poincare plots and Lomb-Scargle periodograms analyses in HRV studies in children, both in physiological and clinical investigations. Another implication comes from the interpretation of the CL parameters –it appears that with increasing maturity the contribution of long-time variability to the total HRV increases at the cost of the short-term variability in healthy children.

ETHICS STATEMENTS

Poznan University of Medical Science Bioethical Committee Parents of all children as well as children of at least 7 years old themselves gave their informed consent for the enrollment to the study. All tests were non-invasive, painless and were additive to the standard examination.

AUTHOR CONTRIBUTIONS

MS, KG, and WB: substantial contributions to the selection of children, their clinical evaluation, acquisition of Holter ECGs and their analysis, interpretation of data for the work; TK, JP, and PG: Signal analysis, computation of HRV parameters, data interpretation. WB, JP, AW, and PG: the conception or design of the work, drafting the work or revising it critically for important intellectual content, final approval of the version to be published, work on corrections of the manuscript and replies to reviewers comments; WB: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. PG: corresponding author.

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Relation of Heart Rate and its Variability during Sleep with Age, Physical Activity, and Body Composition in Young Children

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Background: Recent studies have claimed a positive effect of physical activity and body composition on vagal tone. In pediatric populations, there is a pronounced decrease in heart rate with age. While this decrease is often interpreted as an age-related increase in vagal tone, there is some evidence that it may be related to a decrease in intrinsic heart rate. This factor has not been taken into account in most previous studies. The aim of the present study was to assess the association between physical activity and/or body composition and heart rate variability (HRV) independently of the decline in heart rate in young children.

Methods: Anthropometric measurements were taken in 309 children aged 2–6 years. Ambulatory electrocardiograms were collected over 14–18 h comprising a full night and accelerometry over 7 days. HRV was determined of three different night segments: (1) over 5 min during deep sleep identified automatically based on HRV characteristics; (2) during a 20 min segment starting 15 min after sleep onset; (3) over a 4-h segment between midnight and 4 a.m. Linear models were computed for HRV parameters with anthropometric and physical activity variables adjusted for heart rate and other confounding variables (e.g., age for physical activity models).

Results: We found a decline in heart rate with increasing physical activity and decreasing skinfold thickness. HRV parameters decreased with increasing age, height, and weight in HR-adjusted regression models. These relationships were only found in segments of deep sleep detected automatically based on HRV or manually 15 min after sleep onset, but not in the 4-h segment with random sleep phases.

Conclusions: Contrary to most previous studies, we found no increase of standard HRV parameters with age, however, when adjusted for heart rate, there was a significant decrease of HRV parameters with increasing age. Without knowing intrinsic heart rate correct interpretation of HRV in growing children is impossible.

Keywords: heart rate variability, children, cardiovascular health, cardiac autonomic nervous system, growth, SPLASHY

INTRODUCTION

Cardiovascular (CV) risk factors develop in early childhood. They may alter autonomic balance which seems to be associated with reduced heart rate variability (HRV; Zhou et al., 2012; Vrijkotte et al., 2015). On the other hand, physical activity (PA) and a high exercise capacity appear to positively influence indices of HRV in children and adolescents by increasing HRV (Nagai et al., 2004; Brunetto et al., 2005; Gutin et al., 2005; Buchheit et al., 2007; Krishnan et al., 2009; Michels et al., 2013; Radtke et al., 2013). Parameters of HRV as a surrogate measure of autonomic nervous function have been found to be predictors for cardiovascular mortality in cardiovascular disease (Bigger et al., 1992; Fei et al., 1996; Dekker et al., 2000). Therefore, and due to the ease of application and its non-invasive nature, HRV has become popular in children for the assessment of cardiovascular health.

Previously performed studies in children have used short measurements of 5–10 min during the day (Michels et al., 2013; Seppälä et al., 2014; Gasior et al., 2015) or 24-h recordings (Silvetti et al., 2001; Zhou et al., 2012). Short-term recordings are often poorly standardized because children cannot relax and stay motionless on demand, while 24-h monitoring is highly dependent on the duration of physical activity, sleep, and resting (Montgomery-Downs et al., 2006). An appropriate recording condition could be provided by deep sleep (Brandenberger et al., 2005), a highly standardized state with low sympathetic activity, and stationary HRV data undisturbed by environmental stimulants. Despite these advantages, nighttime recordings have rarely been used in children (Finley and Nugent, 1995; Goto et al., 1997; Radtke et al., 2013), possibly due to the confounding by sleep depth (Villa et al., 2000). Although determination of deep sleep usually requires recording of an electroencephalogram (EEG) and traditional sleep staging, this was not possible in the present setting. Therefore, we developed our own algorithm to detect deep sleep phases.

Pediatric studies that have reported HRV data have generally reported an increase in HRV markers of vagal tone with increasing age up to age 6 (Finley and Nugent, 1995; Goto et al., 1997) or 10 years (Villa et al., 2000; Silvetti et al., 2001; Michels et al., 2013). However, all of these studies have also found an age associated decrease in heart rate (HR). It is questionable whether this decrease can simply be ascribed to an age associated increase in vagal tone or whether it may be related to the substantial growth of the heart during this age period. Some authors have suggested that failure to adjust for changing HR may over- or underestimate HRV responses (Sacha and Pluta, 2008; Billman, 2013; Monfredi et al., 2014). One recent study has adjusted HRV parameters measured in children aged 6–13 years with prevailing HR and has in fact found a decrease in HRV parameters with age (Gasior et al., 2015).

The main aim of the present study was to assess the effect of PA, anthropometric parameters and body composition on linear and non-linear HRV parameters while correcting for the growth associated decline of HR. A secondary aim was to compare different methodologies to measure HRV during sleep in young children.

MATERIALS AND METHODS

Study Design and Participants

The present study was conducted under the umbrella of the Swiss Preschoolers Health Study (SPLASHY, Current Controlled Trials Registry: ISRCTN41045021). SPLASHY is a prospective, multi-center, national study to investigate the effect of stress and physical activity on health in preschool children. The present study is based on cross-sectional data of the baseline assessments in 2014 including healthy preschoolers aged 2–6 years recruited from randomly selected childcare centers in Switzerland. Ethical approval was obtained from the responsible ethical committees of the respective cantons, and the children's parents provided written informed consent in accordance with the Declaration of Helsinki.

Study Procedure

The children were fitted with two chest electrodes and a small device (e-Motion, Mega Electronics, Kuopio, Finland) which records interbeat duration (R-R intervals) at a sampling rate of 1,000 Hz, and a three-dimensional accelerometer validated for preschool children (Pate et al., 2006; Actigraph wGT3x, Shalimar, FL, USA) around the waist. Children and parents/caregivers were instructed in how to continuously wear the monitor over 8 consecutive days except during swimming and showering. The parents were instructed in taking off and reinstalling the R-R interval monitor if children took a shower/bath and in taking it off in the morning following the night recording. Height and weight were measured based on standard procedures and body mass index (BMI) *z*-scores calculated according to World Health Organization (WHO) criteria (http://www.who.int/childgrowth/standards/bmi_for_age/en/). Skinfolds were measured in triplicate on the right body side at four sites (biceps, triceps, subscapular, suprailiac) using a Harpenden caliper. The average of the values for each site was calculated and the sum of the four sites was used for statistical analysis.

HRV Measurement

R-R intervals were further analyzed using Matlab (2014a, The Mathworks, Natick, MA) with a procedure developed specifically for this study. R-R interval recordings and accelerometer data of the same time period were synchronized (**Figure 1**). Deep sleep was approximated by means of two different approaches: firstly, by assuming that young children enter deep sleep within 20 min after sleep onset (Montgomery-Downs et al., 2006), and secondly, based on HRV parameters, since some studies have shown that determination of sleep stages by HRV showed close correspondence to EEG signals (Charloux et al., 1998; Otzenberger et al., 1998; Brandenberger et al., 2001). To allow comparison of our results with the methodology of existing studies who have collected HRV night data irrespective of sleep phase, we have also analyzed a 4-h segment between midnight and 4 a.m. Consequently, of the valid R-R interval recordings, three segments were determined for HRV analysis: (1) a 5-min segment during automatically determined deep sleep with highest percentage of high frequency (HF) power of the HRV frequency spectrum (*high %HF*); (2) a 20-min segment during the first deep sleep phase starting 15 min after sleep onset *(15'aSO)*; and (3) a 4-h segment starting at midnight (*4-h*).

15'aSO

This 20-min time segment was selected 15 min after sleep onset (SO). SO times were automatically determined for each night based on (1) no accelerometer activity and (2) a clear shift toward a lower HR. When no simultaneous valid accelerometer recording was available, SO was determined based on the sudden constant decrease in HR only. Two researchers from the team visually and independently validated the R-R interval signal from the automatically computed SO times. If the automatic SO detection was obviously wrong, the SO time was changed manually. In a further step, all manually adjusted SO times and unclear data were discussed in a group of four researchers and a decision was made by agreement of all. ECG signal artifacts (i.e., due to removal of the device or loss of electrodes during sleep) and data files with unidentifiable SO time were excluded.

High %HF

By means of a custom built Matlab procedure, percentage HF power of total power (TP) (for explanation see chapter "HRV analysis" below) was calculated from 5-min windows moved by 30 s over the whole night. Previous studies have shown specific HRV characteristics in deep sleep with stationary and uncorrelated successive R-R intervals in deep sleep and a high percentage of HF power (Brandenberger et al., 2005; Shinar et al., 2006), which has been confirmed also in children (Villa et al., 2000; Ferri et al., 2002). We developed an algorithm to identify the first segment with HF exceeding 90% of TP during a minimum of 10 min, which was chosen for HRV analysis. If no segment was found using a threshold of 90%, the threshold was lowered to 80% if necessary (<10% of the recordings). A 5-min segment was placed in the middle of the identified segment and used for the HRV analysis.

4-h Segment

A *4-h* segment was selected from 12 to 4 a.m. regardless of SO time or sleep/wake phases, in analogy to previous studies (Finley and Nugent, 1995; Goto et al., 1997) for comparative reasons.

HRV Analysis

The following time domain parameters were used for analysis: HR (beats.min^{-1}), the square root of the mean squared differences of adjacent R-R intervals (RMSSD, ms) and the standard deviation of all R-R intervals (SDNN, ms). For spectral analysis, R-R intervals were interpolated using a cubic spline interpolation method and then resampled at 4 Hz. We applied an advanced smoothness prior approach for detrending of R-R intervals with a smoothing parameter of $\lambda = 500$, which corresponds to a cut-off frequency of 0.035 Hz (Tarvainen et al., 2002). We used an artifact correction algorithm that eliminated R-R intervals in case of deviations of 30% or more of adjacent R-R intervals and replaced them using a cubic-spline interpolation. Power spectral density was then calculated using Fast Fourier Transformation. Frequency domain parameters were total power (TP, ms^2, 0–0.4 Hz), low-frequency power (LF, ms^2, 0.04–0.15 Hz), high-frequency power (HF, ms^2, 0.15–0.4 Hz), and the LF/HF power ratio (Camm, 1996). HF and LF in normalized units are not reported due to their redundancy with LF/HF power ratio. Markers for vagal tone are generally accepted to be RMSSD and HF (Camm, 1996). Additionally, detrended fluctuation analysis (DFA) was performed (Rajendra Acharya et al., 2006). The short-term fractal scaling exponent alpha 1 was calculated for window sizes between 4 and 11 beats and used for analysis.

Physical Activity

Accelerometery data was recorded at a sampling rate of 30 Hz. Periods of 20 min of continuous zero values were interpreted as not worn and removed. A minimum of 4 days with 10 h of wearing time on each day were required for inclusion in the data analysis. PA data recorded between 7 a.m. and 9 p.m. were included in the analysis that defined total daily PA (counts.min^{-1}), light PA (LPA, min.day^{-1}), moderate-to-vigorous PA (MVPA, min.day^{-1}), vigorous PA (VPA, min.day^{-1}), total PA (TPA, min.day^{-1}), and sedentary time (ST, min.day^{-1}) using the cutpoints by Butte et al. (2014).

Statistical Analysis

Statistical analysis was performed using the software R (Version 3.2.3, R Core Team, 2015). Normality of the data was visually assessed using QQ-plots. Differences between boys and girls in anthropometric parameters and physical activity were assessed using unpaired *t*-tests. HRV parameters of the three segments were compared pair-wise using Wilcoxon signed-rank testing. We decided not to use Friedman's test for repeated measures because of the list-wise omission of cases when one segment was missing. Spearman correlations were performed for corresponding HRV parameters of the different segments. Effects of body composition and physical activity on HRV parameters of all three segments were assessed using linear

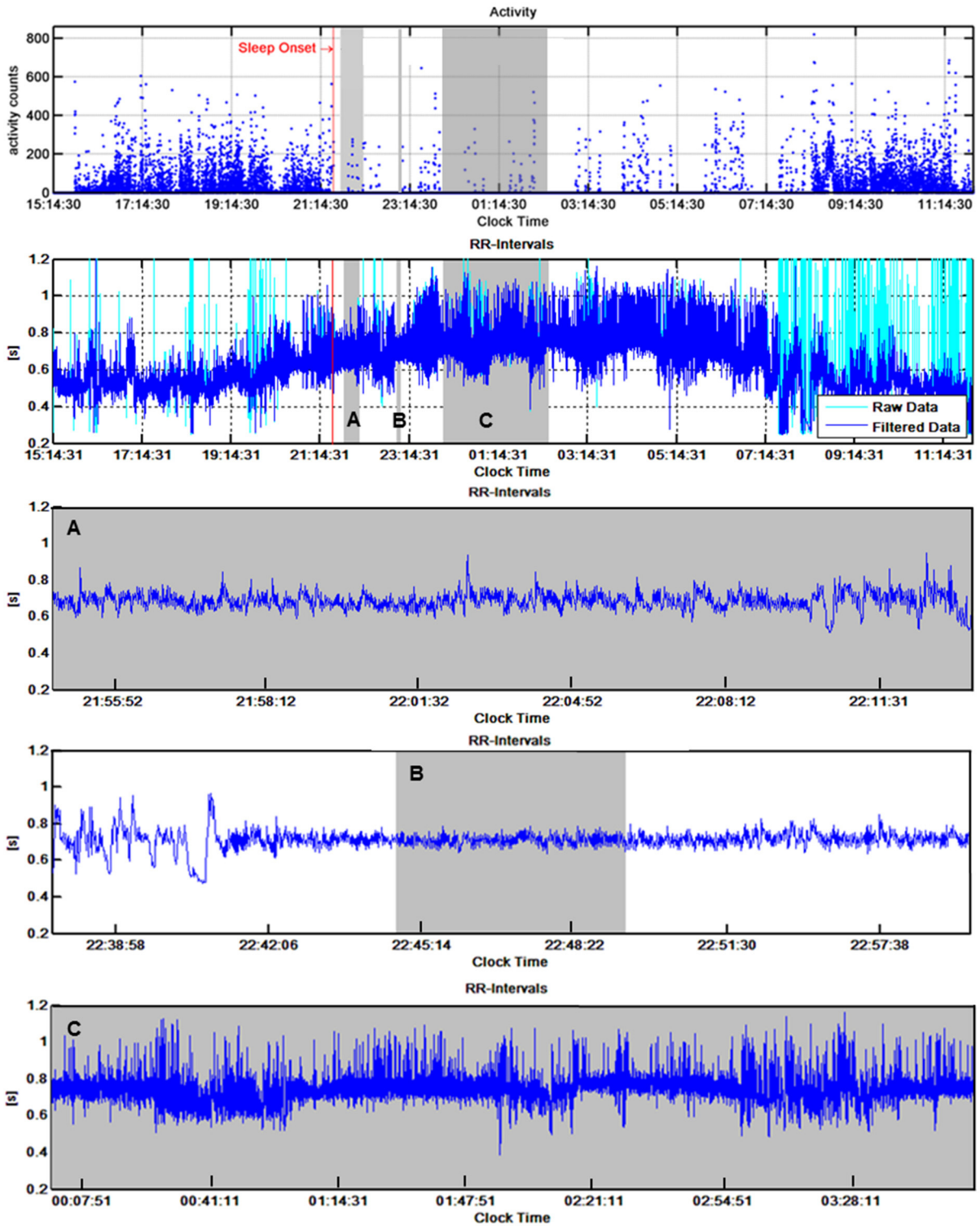

FIGURE 1 | Typical example of accelerometer (top panel) and R-R interval recordings (second panel from top) of one child. The three bottom panels show the R-R intervals of the three selected segments as indicated by shading: the 15 min after sleep onset segment (*15'aSO*, **A**), the high % high frequency segment (high %HF, **B**), and the 4-h segment **(C)**. Please note that the time scales on the x-axes vary.

regression models. Analyses were performed for dependent variables HR, lnRMSSD, lnSDNN, and DFA alpha 1 and independent anthropometric parameters entered together with HR, as well as activity parameters entered together with HR and age. Non-normally distributed HRV variables were log transformed. Models were tested with regard to satisfaction of underlying statistical assumptions such as normal distribution of residuals and homoscedasticity. A Spearman correlation matrix with all variables entered into the models was performed to assess the potential presence of collinearity and suppressor effects. A $p < 0.05$ was considered statistically significant. A sub-analysis was performed on the basis of nine sub-groups: Children were divided into three approximately equally sized (n $\cong$ 103) age groups (young: 2–3.49 years; mid: 3.5–4.19 years; old: 4.2–6 years) as well as three equally sized HR groups (low: <83 beats.min^{-1}; mid 83–91 beats.min^{-1}; high: >91 beats.min^{-1}). Of the nine resulting groups, the smallest was selected ($n = 27$). From the remaining groups, 27 children were randomly selected within each group. Kruskal–Wallis tests were performed between age groups for all HRV parameters. Also, Kruskal–Wallis-tests were performed within each age group between HR groups and within each HR group between age groups.

RESULTS

Study Population

We collected data from 476 children attending 84 different childcare centers in Switzerland. Of these, 402 children had overnight ECG measurements of which 325 could be analyzed. Valid accelerometry data were obtained from 435 children. Only children with both, valid overnight ECG and valid daytime accelerometry data, i.e., 309 children, were included in the statistical analysis. The sample included 162 boys and 147 girls with a mean age of 3.9 ± 0.7 years, height of 102.8 ± 6.6 cm, weight of 17.1 ± 2.5 kg, BMI *z*-score of 0.5 ± 0.9, and sum of skinfolds of 25.8 ± 5.5 mm. Total PA was 1428 ± 284 cpm, children spent 236 ± 53, 457 ± 47, 76 ± 35, 5 ± 5 min.day^{-1} in sedentary time, light, moderate-to-vigorous, and vigorous PA, respectively. Boys were taller and heavier, had a smaller sum of skinfolds and a greater volume of TPA and MVPA than girls (all $p \leq 0.02$), while age, BMI, LPA, VPA, and sedentary time did not differ between sexes. Children's anthropometric characteristics as well as physical activity data for the different age groups are shown in **Table 1**.

Comparison of the Three Segments

Out of 309 measured children, 309 *15'aSO* segments could be analyzed. One *high %HF* segment could not be detected due to loss of functioning of the ECG-monitor early in the night and 308 nights could be used for this method. Thirty-three *4-h* segments had to be excluded due to the absence of valid ECG data. HRV parameters analyzed of the three segments are summarized in **Table 2**. Spearman correlation coefficients of at least 0.9 were found for HR, RMSSD and SDNN between the *15'aSO* and the *high %HF* segments (all $p < 0.001$). Despite these high correlations, a significant difference was found for each HRV parameter ($p < 0.01$) due to a small systematic difference (**Table 2**), congruent with a lower HR in the *high %HF* segment (−1.8 ± 4.3%) compared to the *15'aSO* segment. Also, the *high %HF* segment appeared on average 35 ± 51 min later during the course of the night. Spearman correlation coefficients between the *4-h* segments and both other segments were all smaller than 0.6 (all $p < 0.001$).

HRV Parameters and Effects of Age, PA, and Body Composition

HRV data from the *high %HF* segments for the different age groups are reported in **Table 1**. HR of the *high %HF* segments for each age group is shown in **Figure 2**.

Models were conducted for HRV parameters of all three segments. Results of the *15'aSO* segments and *high %HF* segments were comparable, while HRV parameters of the *4-h* segments resulted in non-significant models. Therefore, we only present results from the linear regression models with HRV parameters of the *high %HF* segment (**Table 3**). RMSSD and SDNN had to be log transformed in order to satisfy the requirement of normal distribution and linearity with HR for the linear models. Inverse effects of age (**Figure 2**), height and weight were found on HR as well as on RMSSD and SDNN in the models adjusted for HR (all $p < 0.01$). RMSSD and SDNN decreased significantly with increasing age when adjusted for HR, which is illustrated in **Figure 3** (middle panel). Pearson correlation coefficients of anthropometric (as well as physical activity) parameters with HR were much greater than with RMSSD or SDNN, except for skinfolds that had the same effect size (**Table 4**). To avoid redundancy, Spearman correlations were only performed with HRV parameters of the *high %HF* segments. All HRV parameters were strongly correlated with HR. Parameters of PA showed the closest relationship with age (and consequently height and weight), followed by HR and only weak relationships to ln(RMSSD) and ln(SDNN). There was also a weak inverse relationship between skinfolds and parameters of PA. Of note is the correlation coefficient of 0.98 between ln(RMSSD) and ln(SDNN), implying that the selected segments of deep sleep total variability was dominated by variability of successive beats. The dependency of RMSSD on HR is shown in **Figure 3** (left panel), with HR explaining 43% of the variance in lnRMSSD ($p < 0.001$). MVPA (**Figure 3**, right panel) and TPA (and by trend for VPA $p = 0.053$) as well as skinfold thickness were significantly related to HR (all $p < 0.01$) while showing no significant relation with RMSSD and SDNN. HR decreased significantly with increasing TPA and MVPA even after adjusting for the significant effect of age on HR (**Table 3**, **Figure 3**, right panel). In the sub-analysis based on the nine groups formed with regard to age and HR, a significant effect of HR (all $p < 0.01$) but no effect of age on RMSSD were observed (all $p > 0.25$, and the same applied to all other HRV parameters; **Table 5**, **Figure 4**). Results for frequency domain parameters showed the same effects as for the time domain parameters (HF was consistent with RMSSD and LF with SDNN) and thus, are not reported.

TABLE 1 | Anthropometric, physical activity and HRV data for the different age groups.

	2 years	3 years	4 years	5 years
N [m,f]	19 (5; 14)	158 (58; 49)	108 (58; 50)	23 (13; 10)
Age [years]	2.8 (2.6; 2.9)	3.5 (3.3; 3.7)	4.3 (4.1; 4.6)	5.5 (5.2; 5.9)
Height [cm]	94 (91; 95)	100 (97; 107)	106 (103; 108)	117 (110; 118)
Weight [kg]	14.1 (12.9; 15.8)	16.0 (15.0; 17.5)	17.8 (16.2; 18.9)	20.9 (18.8; 22.9)
BMI [$kg.m^{-2}$]	16.4 (15.7; 17.1)	16.1 (15.4; 16.9)	15.9 (15.3; 16.6)	15.6 (15.0; 16.8)
TPA [$counts.min^{-1}$]	1,321 (1,102; 1,401)	1,357 (1,160; 1,554)	1,489 (1,289; 1,680)	1,571 (1,367; 1,705)
MVPA [$min.day^{-1}$]	54.8 (35.6; 78.2)	60.6 (45.0; 87.4)	83.6 (62.0; 110.0)	101.7(84.3; 114.3)
HR [$beats.min^{-1}$]	87.6 (85.2; 93.26)	86.9 (80.7; 93.4)	84.9 (78.0; 90.2)	80.0 (74.1; 85.8)
RMSSD [ms]	62.3 (36.5; 86.5)	56.7 (35.6; 94.8)	57.3 (37.8; 85.2)	66.0 (38.9; 124.8)
SDNN [ms]	58.9 (32.6; 72.1)	47.2 (31.6; 75.6)	49.9 (33.3; 69.3)	53.5 (32.0; 95.2)
HF [ms^2]	2,526 (754; 3,699)	1,499 (630; 3,983)	1,559 (719; 3,292)	2,051 (718; 6,309)
LF [ms^2]	228 (172; 464)	242 (102; 579)	230 (111; 620)	415 (144; 723)
LF/HF	0.14 (0.08; 0.27)	0.16 (0.10; 0.25)	0.16 (0.10; 0.30)	0.17 (0.10; 0.26)
Total Power [ms^2]	2,961 (902; 4,538)	1,764 (742; 4,652)	1,284 (869; 3,961)	2,474 (824; 7,684)
DFA alpha 1	0.60 (0.52; 0.69)	0.55 (0.46; 0.63)	0.55 (0.44; 0.63)	0.52 (0.41; 0.59)

TPA, total physical activity; MVPA, moderate-to-vigorous physical activity; HR, heart rate; RMSSD, square root of the mean squared differences of adjacent RR intervals; SDNN, standard deviation of RR intervals; HF, high frequency power; LF, low frequency power; DFA, detrended fluctuation analysis.
Data are presented as median (interquartile range). HRV parameters are calculated from the high %HF segments.

TABLE 2 | Median (IQR) of HRV parameters of the different nighttime segments.

	Segment 1 15-min-aSO	Segment 2 High-HF%	Segment 3 4-h	Difference between segment 1 and 2 [%]
HR [$beats.min^{-1}$]	87.4 (81.7; 93.4)	85.7 (79.5; 91.7)[a]	85.6 (80.3; 85.6)	−1.9
RMSSD [ms]	57.4 (37.2; 87.9)	58.5 (39.9; 91.2)[a]	61.5 (42.8; 85.0)	1.9
SDNN [ms]	53.2 (37.2; 72.7)	49.4 (32.5; 72.5)[a]	86.2 (68.0; 106.3)[a,b]	−7.1
HF power [ms^2]	1,558 (658; 3,324)	1,721 (705; 3,691)[a]	1,936 (985; 3,729)[a]	10.5
LF power [ms^2]	405 (231; 947)	250 (106; 608)[a]	1,320 (816; 2,036)[a,b]	−38.1
LF/HF	0.25 (0.15; 0.45)	0.16 (0.10; 0.27)[a]	0.68 (0.49; 0.93)[b]	−36.0
Total Power [ms^2]	2,259 (993; 4,397)	2,028 (826; 4,599)[a]	7,366 (5,759; 9,625)[a,b]	−10.2
DFA alpha 1	0.58 (0.46; 0.69)	0.55 (0.45; 0.64)	0.62 (0.47; 0.81)	−5.2%

IQR, interquartile range; HRV, heart rate variability; HR, heart rate; aSO, after sleep onset; RMSSD, square root of the mean squared differences of adjacent RR intervals; SDNN, standard deviation of the RR intervals; HF, High frequency power; LF, low frequency power; DFA, detrended fluctuation analysis;
[a] $p < 0.01$ *(Wilcoxon signed-rank test) for difference with segment 1;*
[b] $p > 0.01$ *(Wilcoxon signed-rank test) for difference with segment 2.*

DISCUSSION

The results of the present study show a decrease in HR with increasing age and PA, an increase with skinfold thickness, and no increase in HRV parameters including the commonly used markers of vagal tone. When HRV parameters were adjusted for HR there was a decrease in RMSSD and SDNN with age. This is in accordance with one recent study (Gasior et al., 2015) but in contrast to many previous studies (Finley and Nugent, 1995; Goto et al., 1997; Villa et al., 2000; Silvetti et al., 2001; Michels et al., 2013). The adjustment with HR is based on the assumption that intrinsic heart rate declines with age due to the growth of the heart (Cumming and Mir, 1970). Adjusting HRV parameters for HR in children of an age range where large declines in HR occur has a major impact on HRV results and their interpretation (Monfredi et al., 2014; Gasior et al., 2015). Further, there was no association between HR-adjusted HRV parameters reflecting vagal tone with sex, BMI, skinfold thickness, and PA. However, HR itself was related to skinfold thickness and inversely related to PA. Further, by performing a sub-analysis based on groups comparable either with regard to age or HR, we attempted to overcome the problem of HRV dependency on HR. No differences in HRV parameters were observed in the age groups with similar HR (**Table 5**), despite a higher amount of PA in the older age groups (**Table 4**). These results do not support an age-related increase of vagal tone in young children, contrary to what has been suggested previously. Based on the methods used in our study, we cannot identify the exact reasons of the age-related decrease in heart rate, however we propose that structural and morphological changes of the heart during growth may be responsible.

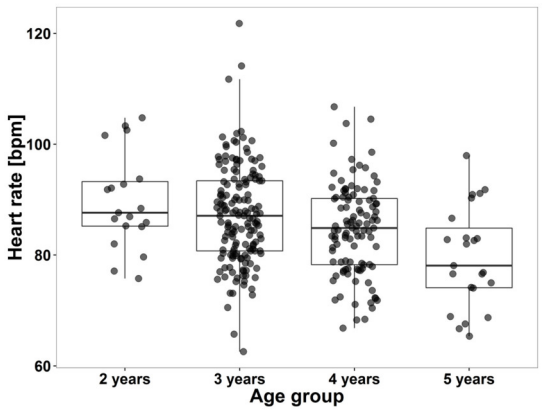

FIGURE 2 | Boxplot showing "deep sleep" heart rates of children according to their age. Box plots show group medians (solid line), IQR (box outline) and spread of data without outliers (whiskers) for each group. The spread of dots with regard to the x-axis reflects younger and older children within each age group. There was a significant difference according to Kruskal–Wallis testing between children aged 2 and 5 years ($H = 17.8$, $p < 0.001$). Parametric linear regression revealed a significant decline of heart rate with increasing age ($r = -0.27$, $p < 0.001$). Data are based on HRV data from the *high %HF* segments.

TABLE 3 | Linear regression models of time domain HRV parameters from the High %HF segment.

	HR Standardized β	ln(RMSSD) Standardized β	ln(SDNN) Standardized β	DFA alpha 1 Standardized β
Age	**−0.21‡**	**−0.16‡**	**−0.15‡**	0.09
HR	–	**−0.70‡**	**−0.62‡**	**0.49‡**
Height	**−0.18‡**	**−0.13‡**	**−0.12†**	0.02
HR	–	**−0.68‡**	**−0.61‡**	**0.48‡**
Weight	**−0.13†**	**−0.14‡**	**−0.14‡**	0.02
HR	–	**−0.67‡**	**−0.61‡**	**0.48‡**
BMI	0.06	−0.04	−0.06	0.01
HR	–	**−0.65‡**	**−0.59‡**	**0.47‡**
$BMI_{Z-score}$	0.04	−0.05	−0.06	0.02
HR	–	**−0.65‡**	**−0.59‡**	**0.47‡**
Skinfolds	**0.28‡**	−0.01	−0.06	−0.08
HR	–	**−0.63‡**	**−0.57‡**	**0.49‡**
Sex	0.09	0.01	0.02	0.08
HR	–	**−0.65‡**	**−0.59‡**	**0.48‡**
TPA	**−0.17‡**	−0.04	0.03	0.01
HR	–	**−0.69‡**	**−0.63‡**	**0.49‡**
Age	**−0.17‡**	**−0.15‡**	**−0.14‡**	0.01
MVPA	**−0.22‡**	−0.05	−0.03	0.01
HR	–	**−0.7‡**	**−0.63‡**	**0.49‡**
Age	**−0.14‡**	**−0.14‡**	**−0.14‡**	0.08

HRV, heart rate variability; HR, heart rate; ln, natural logarithm; RMSSD, square root of the mean squared differences of adjacent RR intervals, SDNN, standard deviation of the RR intervals; DFA, detrended fluctuation analysis; BMI, body mass index; TPA, total physical activity; MVPA, moderate-to-vigorous physical activity. Linear regression models were applied for the dependent variables heart rate (HR), ln(RMSSD) and ln(SDNN) and independent anthropometric parameters entered together with HR, as well as physical activity parameters entered together with HR and age. Bold numbers indicate statistically significant standardized β-coefficients.
†$p \leq 0.05$, ‡$p \leq 0.01$.

There are a number of previous studies that have assessed HRV in young children. However, only some of them have reported nocturnal HRV measurements (Finley and Nugent, 1995; Goto et al., 1997; Massin and von Bernuth, 1997). Two studies have described an increase in HRV parameters reflecting vagal tone (RMSSD, HF, and to a lesser extent SDNN and TP) from infancy to age 6 years and a slow decrease of these parameters thereafter (Finley and Nugent, 1995; Goto et al., 1997). Several more studies have found an increase in HRV markers of vagal tone in children older than 6 years (for a review see Eyre et al., 2014). Most of these studies (Finley and Nugent, 1995; Goto et al., 1997; Michels et al., 2013) did not control for HR despite the recognized importance of correcting HRV parameters with HR (Sacha and Pluta, 2008; Billman, 2013). The only study that has adjusted HRV parameters for prevailing HR has found no change in HRV parameters with age in children aged 6–13 years (Gasior et al., 2015). In accordance with our results, they found a strong decrease of HR with age and also an age-related decrease in HR-adjusted HRV parameters. In another cohort of 460 boys and girls aged 5–12 years a significant increase in RMSSD and HF was found with age without adjusting models for HR (Michels et al., 2013). After adjustment with HR, the authors also found RMSSD and HF power to be inversely related to age (pers. Comm.) which is in accordance with our and Gasior and colleagues' results (Gasior et al., 2015). A further study found both age and HR to be independently related to HRV parameters as well as to each other (Massin and von Bernuth, 1997). They pointed out the difficulty of a correct interpretation of autonomic nervous activity because of an interdependence between age, HR, respiration frequency, blood pressure, and HRV.

It is likely that in growing children, HR decreases as a consequence of different factors such as the growing heart which grows proportionally to height; (St John Sutton et al., 1982; O'Leary et al., 2015), larger total blood volume and increasing blood circulation time (Morse et al., 1947). An inverse relationship between body mass and HR has been found across different mammal species [for summary see Dobson et al. (Dobson, 2003)]. HRV has been found to be inversely related to HR, with larger R-R intervals allowing for larger variation (Billman, 2013; Dvir et al., 2013; Monfredi et al., 2014). The dependency of HRV on HR has recently been described showing that HRV is inextricably linked to HR in an exponential manner (Zaza and Lombardi, 2001), for which biophysical properties have been suggested to be responsible (Monfredi et al., 2014). The importance of correcting for prevailing HR to reflect cardiac autonomic activity more closely has been pointed out by a study on different autonomic interventions (Billman, 2013). Taking this into account, values of HRV parameters may not reflect activity of the cardiac autonomic nervous system across different age ranges in growing children. Rather, the often documented increase in HRV parameters with age mainly reflects the age-related decrease in HR, which, between age 2 and 6 years, has been found to be ∼20

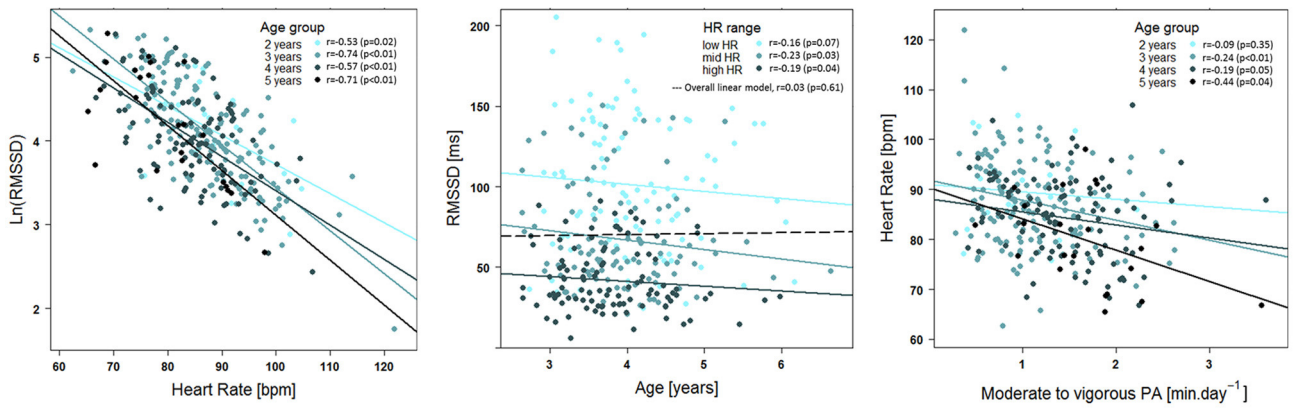

FIGURE 3 | Raw data scatter plots. Linear relationship between lnRMSSD and HR for children according to age (marked with different colors) is shown in the left panel. RMSSD [ms^2] was log transformed to receive a linear relationship with HR. Lines indicate linear regressions for each age group. The weak but significant decline of RMSSD with age for three different HR ranges (low HR: <83 beats.min^{-1} medium HR: 83–91 beats.min^{-1}, high HR: >91 beats.min^{-1}), reflecting the result of the HR-adjusted mixed linear model for lnRMSSD with age, is illustrated in the middle panel. The overall regression line (dashed line) represents the linear regression of RMSSD vs. age without adjustment for HR. Effect of volume of daily moderate to vigorous physical activity on resting HR for individual age classes is shown in the right panel. Solid lines represent linear regressions for each age group. All plots are based on HRV data from the *high %HF* segments. RMSSD, root mean squared successive differences; ln, natural logarithm; HR, heart rate; PA, physical activity; HF, high frequency.

TABLE 4 | Pearson correlation coefficients between anthropometric variables, PA variables and HRV parameters.

	HR	ln(RMSSD)	ln(SDNN)	DFA	Age	Height	Weight	BMI	Sex	TPA	MVPA	Skinfolds
HR	–											
ln(RMSSD)	**−0.61**^{b}	–										
ln(SDNN)	**−0.57**^{b}	**0.98**^{b}	–									
DFA	**0.47**^{b}	**−0.28**^{b}	**−0.17**^{b}	–								
Age	**−0.27**^{b}	0.03	0.03	−0.05	–							
Height	**−0.24**^{b}	0.01	−0.01	−0.10	**0.80**^{b}	–						
Weight	**−0.17**^{b}	−0.03	−0.05	−0.07	**0.60**^{b}	0.83	–					
BMI	0.06	−0.05	−0.05	0.03	**−0.17**^{b}	−0.07	0.49	–				
Sex	**0.14**^{b}	−0.06	−0.06	−0.01	**−0.11**^{a}	**−0.21**^{b}	**−0.18**^{b}	0.01	–			
TPA	**−0.25**^{b}	0.10	0.09	**−0.11**^{a}	**0.26**^{b}	**0.26**^{b}	**0.26**^{b}	0.06	**−0.14**^{a}	–		
MVPA	**−0.30**^{b}	**0.11**^{a}	0.10	**−0.11**^{a}	**0.37**^{b}	**0.36**^{b}	**0.33**^{b}	0.01	**−0.22**^{b}	**0.88**^{b}	–	
Skinfolds	**0.14**^{a}	**−0.14**^{a}	**−0.14**^{a}	−0.03	−0.09	0.04	**0.31**^{b}	**0.52**^{b}	**0.26**^{b}	**−0.12**^{a}	**−0.13**^{a}	–

*Abbreviations as in **Table 3**.*
$^a p \leq 0.05$, $^b p \leq 0.01$

beats.min^{-1} (Goto et al., 1997; Fleming et al., 2011; O'Leary et al., 2015).

When HRV is used as a marker of the autonomic nervous activity, correction for HR has to be considered. HRV parameters have been shown to reflect autonomic nervous activity in many previous studies (Pomeranz et al., 1985; Pagani et al., 1986; Malik and Camm, 1993; Goldberger et al., 2001). More recently, studies have suggested the necessity of correcting HRV for HR (Billman, 2013; Sacha, 2013; Monfredi et al., 2014; Gasior et al., 2015). There is no consensus as yet on whether HR correction should be applied or not, probably because the adequate answer depends on the study design, the study population and the research question. While the strong relation between HRV and HR has been shown to be partly due to mathematical (Sacha and Pluta, 2008; Sacha, 2014) and biophysical properties (Zaza and Lombardi, 2001; Monfredi et al., 2014), part of this association is due to the concurrent influence of the autonomic nervous system on both parameters (i.e., increase in vagal activity both increases RMSSD and decreases HR) and correcting with HR could therefore remove observed differences in RMSSD. In our study population of young children between 2 and 6 years, in whom growth is likely to have a substantial effect on structural and morphologic changes of the heart, failing to correct HRV for heart rate may lead to erroneous interpretation of vagal activity with regard to age. Therefore, we suggest that there is no general rule with regard to heart rate adjustment of HRV parameters. We propose that in the absence of knowledge on intrinsic heart rate the interpretation of vagal activity based on "established markers of vagal tone" is risky, to say the least, particularly in populations with large variances with regard to heart structure

TABLE 5 | Sub-analysis: HRV parameters of randomly selected children specifically grouped according to heart rate within age groups.

	Young (2–3.5 years)	Mid (3.5–4.2 years)	Old (4.2–6 years)	*p*-value
HR (beats.min^{-1})	85.8 (80.7; 91.6)	86.7 (79.3; 92.0)	85.5 (78.0; 90.9)	0.49*
RMSSD (ms)	61.9 (35.8; 95.0)	64.6 (40.4; 97.5)	53.9 (33.3; 82.6)	0.33
SDNN (ms)	46.9 (30.6; 74.3)	53.2. (36.5; 81.2)	44.8 (30.4; 67.7)	0.33
HF power (ms^2)	1,479 (570; 3,684)	1,679 (818; 3,524)	1,350 (665; 2,775)	0.59
LF power (ms^2)	236 (106; 557)	296 (121; 751)	221 (100; 574)	0.30
LF/HF	0.19 (0.11; 0.26)	0.17 (0.11; 0.30)	0.15 (0.10; 0.27)	0.48
Total Power (ms^2)	1,947 (711; 4,668)	2,225 (974; 5,100)	1,711 (766; 3,781)	0.71
DFA alpha 1	0.55 (0.45; 0.63)	0.54 (0.46; 0.63)	0.55 (0.45; 0.64)	0.81

*Abbreviations as in **Table 3**.*

Medians (interquartile ranges) are shown. p-values are from Kruskal–Wallis testing. A total of 243 children were randomly selected, 81 in each age group (young, mid, old) of whom 27 in each HR group (low, mid, high HR group).

**Please note there is no difference in HR between the three age groups because groups were specifically formed with regard to HR.*

and/or morphology. The only study that determined intrinsic HR under double blockade of the cardiac autonomic nervous system with propranolol and atropine in 103 infants and children aged 0–16 years with mild to moderate heart defects has found an age related decline in intrinsic HR (Cumming and Mir, 1970). Intrinsic HR was ∼20 beats.min^{-1} higher than control resting heart rate without any blockade at all ages. The authors concluded that the decline in HR was not due to changes in autonomic function. It was suggested that age related changes in the frequency of depolarization in pacemaker tissue were responsible, such as changes in sino-atrial node membrane ion flux or permeability, or alterations in location of the predominant pacemaker cells within the node (Rowland, 2004). Unfortunately, there are no studies on age-related changes in intrinsic HR in healthy children.

We found physical activity to be inversely related to HR but not to HRV parameters in our models adjusted for HR. This is in accordance with what was found in a comparable study in boys, but not in girls (Michels et al., 2013). Numerous studies have assessed the influence of obesity or body composition on HRV in children and adolescents (for a review see Eyre et al., 2014). However, the only study who investigated younger children comparable to our cohort found no association between BMI or percent body fat with HRV parameters (Michels et al., 2013). We observed that increasing skinfold thickness was related to HR, but not to HRV parameters in models adjusted for HR. In older children, several studies have documented reduced HRV markers of vagal tone and increased HR in overweight and obese children compared to normal children (Rabbia et al., 2003; Vanderlei et al., 2010; Altuncu et al., 2012), and more so in obese children with larger amount of central fat (Soares-Miranda et al., 2011).

The close correspondence of deep sleep segments identified by two different methods, one semi-automatically (by decrease in HR und cessation of activity) and one by an algorithm based on high percentage of HF power indicates a reliable identification of deep sleep. The systematic difference between the two segments was most likely due to the later occurrence of the automatically detected segment with an already lower HR. In contrast, HRV during the *4-h* segment was more weakly related to those of deep sleep. The reason for this may be the greater proportion of REM-sleep in the second half of the night (Montgomery-Downs et al., 2006), which is supported by the higher values of SDNN, LF power, and total power. A 4-h segment of the second half of the night is therefore not suitable as it is very heterogeneous and greatly confounded by individual sleep architecture. On the other hand, our method to identify deep sleep phases by high percentage of HF power (corresponding with a low LF/HF ratio) has already been suggested for adults (Camm, 1996). It does not require additional equipment on top of an ECG monitor and does not depend on transition time from sleep onset to deep sleep.

A limitation of the present study is the missing information on true intrinsic HR of our subjects. Our hypothesis that there is a growth-related decrease in HR is based on the only existing study that has determined intrinsic HR under vagal and sympathetic blockade in children, and more general literature that has related HR to heart size. Therefore, we cannot define the development of vagal activity with age but we stress that interpretation of cardiac autonomic nervous system activity greatly depends on the assumption of the origin of HR decline with age (i.e., that either there is a growth-related decrease of intrinsic HR or that HR declines as a consequence of increased vagal activity). Further, the present results are based on cross-sectional data. Longitudinal data over this age range would have reduced the data variance, however, longitudinal data would most likely also show a reduction in HR and the directly linked increase in vagal markers of HRV, without giving any further explanation as to what the origin of HR decline is. Measurements of skinfold thickness only provide an approximation of body composition. However, they were chosen because of non-invasiveness and higher precision than BMI.

Strengths of the present study are the automatic identification of a deep sleep segment providing stationary HRV data undisturbed by environmental stimulants and with regular respiration frequency which could be an optimal method to assess HRV in young children, and secondly, the adjustment for HR in

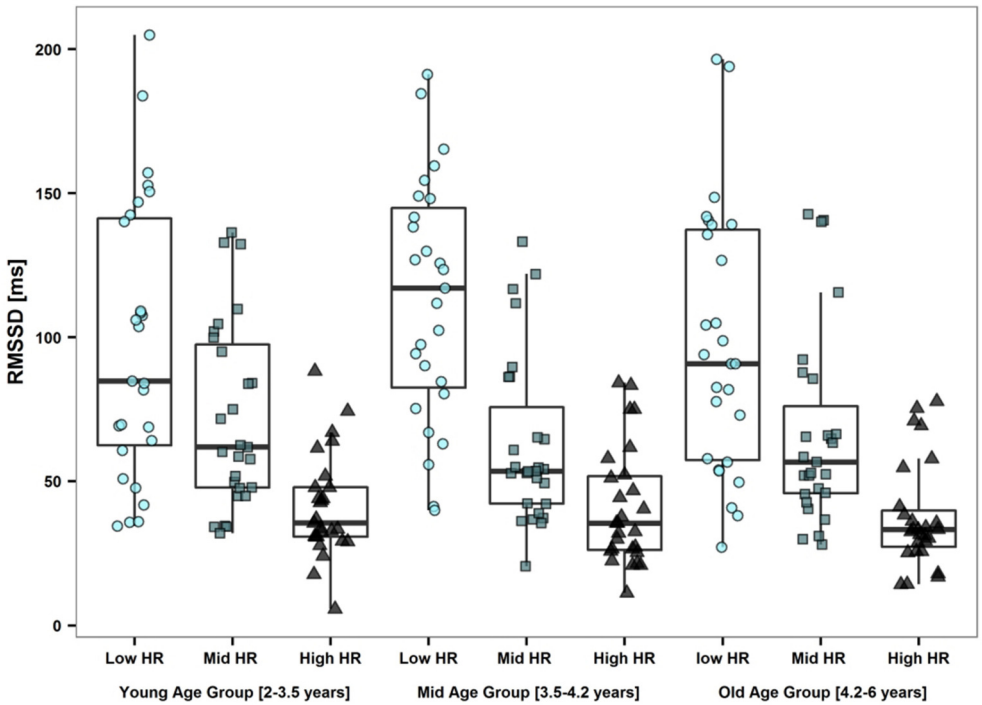

FIGURE 4 | Subanalysis showing reduced data set. A total of 243 children were randomly selected, 81 in each age group (young, mid, old) of whom 27 in each HR group (low, mid, high HR group). No effect of age on RMSSD was observed (all $p > 0.25$). Within the three age groups, RMSSD was significantly different between the HR groups (all $p < 0.01$).

models relating anthropometric and physical activity parameters to HRV markers of vagal tone.

In conclusion, we found no increase of standard HRV parameters with age, however, when adjusted for HR, there was a significant decrease of HRV parameters with increasing age, in accordance with one previous study (Gasior et al., 2015). The age-related decrease of HR, observed across subjects in our cross-sectional data sample, cannot simply be interpreted as an increase in vagal activity. Instead, at least some of the decrease in HR may be related to growth with an increase in heart, vessel size, and blood volume leading to a decrease in intrinsic heart rate. Whether the observed decrease in HR-adjusted HRV parameters reflects a true decrease in vagal activity between age 2 and 6 years remains to be elucidated in longitudinal studies using direct measurement of vagal activity. Thus, in the absence of available measurements of intrinsic heart rate, HRV changes during growth have to be interpreted with caution.

AUTHOR CONTRIBUTIONS

DH, PE, SK, TRa, PA, MW, JP, OJ, SM: designed research of the substudy; DH, PE, TRa, TRu, AW, PA, SK performed data analyses; DH, PE: performed statistical analyses; DH, PE, TRa, SK, PA: wrote and commented the manuscript; NM, TK, KS, CL, ES, AZ, AA, AW, TRa: contributed to data collection. All authors approved the final version of the manuscript.

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Heart Rate Is a Better Predictor of Cardiorespiratory Fitness Than Heart Rate Variability in Overweight/Obese Children: The ActiveBrains Project

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Cardiac autonomic function can be quantified through mean heart rate (HR) or heart rate variability (HRV). Numerous studies have supported the utility of different HRV parameters as indicators of cardiorespiratory fitness (CRF). However, HR has recently shown to be a stronger predictor of CRF than HRV in healthy young adults, yet these findings need to be replicated, in other age groups such as children. Therefore, this study aimed: (1) to study the associations between indicators of cardiac autonomic function (HR, standard and corrected HRV parameters) and CRF in overweight/obese children; and (2) to test which of the two indicators (i.e., HR or HRV) is a stronger predictor of CRF. This study used cross-sectional baseline data of 107 overweight/obese children (10.03 ± 1.13 years, 58% boys) from the ActiveBrains project. Cardiac autonomic indicators were measured with Polar RS800CX®. CRF was assessed using a gas analyzer while performing a maximal incremental treadmill test. Correlations and stepwise linear regressions were performed. Mean HR and standard HRV parameters (i.e., pNN50, RMSSD, and SDNN) were associated with CRF (r coefficients ranging from −0.333 to 0.268; all $p \leq 0.05$). The association of HR with CRF persisted after adjusting for sex, peak height velocity (PHV), adiposity moderate-to-vigorous physical activity, energy intake and circadian-related variable intradaily variability of activity patterns whilst for HRV parameters (i.e., pNN50, RMSSD, and SDNN) disappeared. Stepwise linear regression models entering HR and all HRV parameters showed that mean HR was the strongest predictor of CRF ($\beta = -0.333$, $R^2 = 0.111$, $p < 0.001$). Standard and corrected HRV parameters did not provide additional value to the coefficient of

determination (all $p > 0.05$). Our findings suggest that HR is the strongest indicator of CRF. It seems that quantification of HRV parameters in time and frequency domain do not add relevant clinical information about the cardiovascular health status (as measured by CRF) in overweight/obese children beyond the information already provided by the simple measure of HR.

Keywords: parasympathetic, sympathetic, heart rate variability, treadmill, adiposity, youth

INTRODUCTION

A number of comorbidities linked to childhood obesity could be explained by their altered cardiac autonomic functions (Thayer and Lane, 2007; Zhou et al., 2012). Particularly, overweight/obese children and adults usually present a decreased parasympathetic activity (Gutin et al., 2000; Nagai et al., 2003; Rodríguez-Colón et al., 2011; Triggiani et al., 2017) which, in turn, is related to a higher risk of cardiovascular disease (CVD) (Lahiri et al., 2008). Therefore, the quantification of cardiac autonomic function is a matter of interest in overweight/obese populations.

Heart rate (HR) and heart rate variability (HRV) have been proposed as indicators of the cardiac autonomic nervous system functioning (Koizumi et al., 1985; Task Force, 1996; Lahiri et al., 2008). Specifically, HR reflects the frequency of heart beats per minute. HRV refers to the variability in time intervals between consecutive heart beats, namely between the R peaks registered in the electrocardiogram (ECG) (Task Force, 1996). Both HR and HRV have been used as predictors of CVD, showing that higher HR and lower HRV at rest are related to a higher CVD risk (Kannel et al., 1987; Thayer and Lane, 2007; Trimmel et al., 2015; Dilaveris and Tousoulis, 2018).

A good indicator of the CVD risk in adults is cardiorespiratory fitness (CRF) (Ortega et al., 2016; Ross et al., 2016; Tikkanen et al., 2017), which is also known as a powerful marker of health in children and adolescents (Ortega et al., 2008). In this context, HR is inversely associated with CRF (Kang et al., 2017), but based on a recent systematic review, associations between HRV and CRF are inconsistent in children and adolescents (Oliveira et al., 2017). For example, some studies have found positive associations between HRV and CRF in obese youth (Gutin et al., 2005; Michels et al., 2013; Da Silva et al., 2014), whereas others did not find differences in HRV parameters between 3 groups of healthy adolescents with different levels of CRF (Brunetto et al., 2005). In fact, Oliveira et al. concluded that several methodological differences between studies could contribute to these differences and that relevant studies in pediatric population are currently lacking.

Importantly, HRV reveals a non-linear inverse association with average HR (or direct association with average R-R interval), and consequently some clinical value of HRV must originate from average HR, – to elucidate this issue one should liberate HRV from the influence of HR (Sacha, 2013, 2014a; Sacha et al., 2013a; Sacha and Pluta, 2008). To overcome this HRV dependence on HR, a correction procedure has been proposed which is based on calculating the ratios between HRV parameters and different powers of their corresponding mean R-R interval (Sacha et al., 2013b). Of note, by employing this procedure, the HRV prediction capacity after myocardial infarction has been improved (Sacha, 2014b) and it was also possible to show that HR is a better predictor of CRF than HRV in young adults (Grant et al., 2013), although little is known on how it works in overweight/obese children.

Thus, the aims of this study were: (1) to explore the associations between indicators of cardiac autonomic function (HR, standard and corrected HRV parameters) and CRF; and (2) to test which of the two indicators (i.e., HR or HRV) is a stronger predictor of CRF in overweight/obese children.

MATERIALS AND METHODS

Participants and Study Design

This cross-sectional study used baseline data from the ActiveBrains project[1] (Clinical Trial: NCT02295072). A total of 107 overweight/obese children (10.03 ± 1.13 years, 58% boys) were included in the present study. Information about the methodology and protocol can be found elsewhere (Cadenas-Sánchez et al., 2016). This study was conducted according to the Declaration of Helsinki. The protocol was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). All parents had received information about the study and gave written informed consent in accordance with the Declaration of Helsinki.

Anthropometry and Weight Status

Body weight and height were measured to the nearest 0.1 kg and cm with an electronic scale and a stadiometer, respectively (SECA instruments, Germany, Ltd). Body mass index (BMI) was calculated as kg/m^2 and the participants were classified as overweight, mild obesity or severe/morbid obesity according to the sex-and-age specific international BMI standards (World Obesity Federation, formerly named International Obesity Task Force) (Cole and Lobstein, 2012; Bervoets and Massa, 2014). Body fat percentage was assessed with dual-energy X-ray Absorptiometry (DXA, discovery densitometer from Hologic). All DXA scans and analyses were performed using the GE encore software (version 4.0.2) and were completed following the same protocol by the same evaluator. The positioning of the participants and the analyses of the results were undertaken following recommendations from the International Society of Clinical Densitometry (Crabtree et al., 2014).

[1] http://profith.ugr.es/activebrains?lang=en

Physical Activity

Physical activity (PA) levels were estimated from the triaxial accelerometers ActiGraph GT3X+ (ActiGraph, Pensacola, FL, United States). Accelerometers were initialized to capture and store accelerations at 100 Hz and placed on non-dominant wrist for seven consecutive days. We processed the raw accelerations in the GGIR R package (Van Hees et al., 2013). We considered every participant wearing the accelerometer a minimum of 16 h per day during at least 3 weekdays and 1 weekend day as recommended elsewhere (Migueles et al., 2017, 2019). The daily time spent in moderate-to-vigorous PA intensity (i.e., >3 metabolic equivalents) was estimated using previously proposed cut-points for children (Hildebrand et al., 2014).

Energy Intake

Energy intake was obtained by means of two non-consecutive 24-h recalls during a time span of a week by trained dietitians-nutritionists. The presence of parents or legal guardians was obligatory for the collection of dietary data due to the difficulty for children to remember recipes or amounts of foods. A book with pictures of different food servings and sizes was used to help the participants to estimate the amount of food consumed. The Diet software (Xyris Software, Brisbane, Australia), supported by the Spanish Association of Dietetics and Nutritionists, was used to calculated dietary data.

Circadian-Related Variable Based on Fragmentation of Daytime Activity Patterns: Intradaily Variability

We calculated non-parametric indexes described by Van Someren et al. (1999) to characterize activity patterns. The circadian-related variable selected was fragmentation of daytime activity, measured by intradaily variability (IV), due to has been related to cardiorespiratory fitness and adiposity in adolescents (Garaulet et al., 2016). This circadian-related variable indicates the frequency of changes between high and low activity. Its values oscillated between 0, when the wave was perfectly sinusoidal, and 2, when the wave was as Gaussian noise.

Heart Rate and Heart Rate Variability

Participants were placed supine for 10 min in a quiet and comfortable room between 9 a.m. and 12 p.m. Supine position has shown a higher reliability for the HRV measurement in children than sitting or standing (Silva et al., 2016). The POLAR RS800CX (Polar Electro Oy Inc., Kempele, Finland) recorded HRV during 10 min at a sampling frequency of 1000 Hz. This HR monitor is valid (Gamelin et al., 2008) and reliable when compared with electrocardiography (Vasconcellos et al., 2015) for the HRV assessment in children and adolescents. Participants were encouraged to keep relaxed, breathe normally and do not speak or move during the evaluation.

The indicators of cardiac autonomic function were HR and HRV parameters. For HRV analyses, we derived time and frequency-domain HRV parameters from the normal R-R intervals after excluding the extreme values using an automatized "low filter" in the Kubios software (HRV analysis, University of Eastern Finland) (Niskanen et al., 2004; Tarvainen et al., 2014). The R-R intervals series were detrended using the smoothness prior method with alpha set at 500 and a cubic interpolation at the default rate of 4 Hz. Out of the 10 min recorded, the middle 5 min (i.e., from 3 to 8 min) were visually checked for quality (i.e., normal distribution of the R-R intervals, no large R-R interval outliers and R-R intervals equidistance and minimal variation), and a different period of 5-min was selected when necessary. Time- and frequency-domain HRV parameters were selected based on the Guidelines of Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (Task Force, 1996). In the time-domain, we computed the squared root of the mean of the sum of the squares of successive normal R-R interval differences (RMSSD) and the percentage number of pairs of adjacent normal R-R intervals differing by more than 50 ms in the entire recording (pNN50), both as indexes of parasympathetic activity. We also computed the standard deviation of all normal R-R intervals (SDNN). In the frequency-domain, we performed spectral analyses using the non-parametric fast fourier transformation algorithm (FFT), with Welch's periodogram method (i.e., 50% overlap Hanning window as pre-processing technique and calculating area under the curve with an integration). We derived total power of HRV spectrum (TP), the power in the high (HF) and the low frequency (LF) (TP: 0–0.4 Hz; HF: 0.15–0.4 Hz; LF: 0.04–0.15 Hz) in absolute units (ms^2). HF and the LF/HF ratio were used in the analyses as indicators of parasympathetic activity and sympatho-vagal balance, respectively (Task Force, 1996), although physiological significance of LF and LF/HF ratio is not clear (Goldstein et al., 2011; Billman, 2013).

To remove the HRV dependence on HR, we calculated the corrected HRV parameters proposed by Sacha et al. (2013b) based in two assumptions: (i) If HRV parameters were negatively correlated with HR, correction procedure consisted in calculating ratios between HRV parameters and different powers of their corresponding mean normal R-R interval; (ii) if HRV parameters were positively correlated with HR, the correction procedure was performed by multiplying HRV parameters by the adequate powers of mean normal R-R interval as follows: $RMMSD_c = RMSSD/meanRR^{3.7}$, $pNN50_c = pNN50/meanRR^{5.3}$, $SDNN_c = SDNN/meanRR^{2.8}$, FFT $TP_c = TP/meanRR^{6.3}$, FFT $HF_c = HF/meanRR^{6.3}$, FFT $LF_c = LF/meanRR^{5.0}$, FFT $LF/HF_c = LF/HF \times meanRR^{1.8}$.

Cardiorespiratory Fitness

Cardiorespiratory fitness was assessed using a gas analyzer (General Electric Corporation) while performing a maximal incremental treadmill test (HP-Cosmos ergometer) adapted for low-fit children (Davis et al., 2012). The incremental test consisted of walking on a treadmill at a constant speed (4.8 Km/h) starting at a 6% slope with grade increments of 1% every minute until volitional exhaustion. Children were highly encouraged to walk as long as possible. Maximal peak oxygen consumption (VO_2peak, ml/kg/min), HR (beats/min) and respiratory exchange ratio (RER) were continuously recorded each 10 s, whilst the rating of perceived exertion

(RPE) scale was reported at the end of each 1 min stage using children's OMNI scale ranging from 0 to 10 (Suminski et al., 2008). VO2max was confirmed when meeting 3 out of 4 following criteria: achieving >85% of aged-predicted HRmax, a RER of ≥1.10, volitional fatigue (i.e., >8 points in the OMNI scale) and a plateau in the oxygen consumption during the last two exercise work rates (<2.0 ml/kg/min). We performed analysis with the complete sample that performed the treadmill test (VO_2peak; n = 107) and with the sub-sample of children that met at least 3 out of 4 criteria described above (VO_2max; n = 75). As conclusions were the same in both occasions, we presented here the results analyzing the complete sample with VO_2peak (n = 107).

Demographic Variables

Peak height velocity was assessed as an accurate and discriminant measure of maturational status (Malina et al., 2015). PHV was obtained from anthropometric variables (weight, height, and seated height) using Moore equations (Moore et al., 2015). Years from PHV offset were calculated by subtracting the age of PHV from the chronological age.

Socioeconomic status was assessed by the parental education level. Both father and mother self-reported their maximum educational level (i.e., elementary school, middle school, high school and university degree completed). Parents responses were then combined as follows: none of the parents had a university degree, one of the parents had a university degree or both parents had a university degree.

Statistical Analyses

Descriptive data are presented as frequency and percentage for categorical variables. Kolmogorov-Smirnov test and a visual inspection of histograms were performed to assess the normality of data distribution. Variables that exhibited normal distribution were presented as mean and standard deviation (mean ± SD). Standard HRV parameters did not exhibit normal distribution and were presented as median and interquartile range. For analytical purpose, normal scores of standard and corrected HRV parameters were calculated using Blom formula (Blom, 1958).

In order to test the dependence between HRV and HR parameters, we performed Spearman correlations between standard and corrected HRV parameters and HR. First, the associations of HR, standard and corrected HRV parameters with CRF were studied using Bivariate Pearson correlations. Second, to determine the strongest predictors of CRF, we performed stepwise linear regressions of HR, standard and corrected HRV parameters with CRF. Predictors were included in two different stepwise models. The first model included HR and standard HRV parameters. The second model included HR and corrected HRV parameters. Lastly, we conducted exploratory analyses to test whether the observed associations were independent of potential confounders, such as sex, PHV, adiposity (body fat percentage), moderate-to-vigorous PA, energy intake and circadian-related variable. Analyses were performed using SPSS version 21.0 (IBM Corp., NY, United States). The significance level was set at 0.05.

RESULTS

Table 1 shows the descriptive characteristics of the participants. **Figures 1**, **2** present the Spearman correlations between HRV parameters and HR. Significant correlations found between the standard HRV parameters and HR (r ranging from −0.802 to 0.250; all $p \leq 0.009$) disappeared after the correction procedure (r ranging from −0.120 to 0.033; $p \geq 0.220$).

Table 2 presents Pearson correlations of HR, and time and frequency normal scores (according to Blom, 1958) of standard and corrected HRV parameters with CRF. HR was inversely associated with CRF ($r = -0.333$, $p < 0.001$). The standard HRV parameters in time-domain (RMSSD, pNN50, and SDNN) were positively associated with CRF (r ranging from 0.209 to 0.268, all $p < 0.050$), whereas frequency-domain parameters were not significantly associated with CRF ($p < 0.05$). After correction for the prevailing HR, the RMSSD, pNN50 and SDNN completely lost their association with CRF, i.e., none of the corrected HRV parameter correlated with CRF (all $p > 0.05$).

Table 3 shows stepwise linear regressions of HR and normal scores of standard and corrected HRV parameters with CRF. Out of all variables entered in the model, only HR was selected in the stepwise model as a significant predictor of CRF ($R^2 = 0.111$, $p < 0.001$). The associations of HR with CRF remained significant ($\beta = -0.169$, $p = 0.030$), after adjustment for potential confounders (i.e., sex, PHV, adiposity [body fat percentage], moderate-to-vigorous PA, energy intake and circadian-related variable); whereas the associations of pNN50 and the rest of the HRV parameters with CRF became/remained non-significant ($p > 0.05$) (**Supplementary Table S1**).

DISCUSSION

The main findings of this study were that: (1) HR showed stronger associations with CRF compared with standard and corrected HRV parameters in either time or frequency domains, regardless of sex, maturation, adiposity, moderate-to-vigorous PA, energy intake and circadian-related variable; (2) HR, was the only independent parameter associated with CRF when all the HRV parameters were included in the model. These results suggest that the complex concept of HRV does not seem to add clinically relevant information about the cardiovascular health status, as measured by CRF, in overweight/obese children beyond the information already provided by the "simple" measure of HR.

We found positive associations between standard HRV parameters in time domain (i.e., RMSSD, pNN50, and SDNN) and CRF, with pNN50 being the HRV parameter more strongly associated. In several studies, parasympathetic activity quantified through standard HRV parameters in time and frequency domains (i.e., RMSSD, pNN50, and HF) has been shown to be positively associated with CRF in children and obese adolescents (Gutin et al., 2005; Michels et al., 2013; Da Silva et al., 2014; Redón et al., 2016). However, a recent systematic review showed controversial associations between standard HRV parameters, used as indicators of parasympathetic

activity, and CRF in children and adolescents (Oliveira et al., 2017). The authors concluded that the main limitations of most of the studies were the lack of a detailed explanation of HRV protocol, the use of different protocols to measure CRF and the lack of adjustment for relevant confounders (Oliveira et al., 2017).

TABLE 1 | Descriptive characteristics of the study sample (n = 107).

Variables	Total sample (n = 107)	Boys (n = 62, 58%)	Girls (n = 45, 42%)
Parents with university degree, n (%)			
None of them	71 (66.4)	45 (72.6)	26 (57.8)
One of them	19 (17.8)	9 (14.5)	10 (22.2)
Both of them	17 (15.9)	8 (12.9)	9 (20.0)
Age and maturational status			
Age (years)	10.03 ± 1.13	10.15 ± 1.16	9.88 ± 1.08
PHV offset (years)	−2.26 ± 0.99	−2.65 ± 0.81	−1.71 ± 0.97
Weight status			
Weight (kg)	56.05 ± 11.06	56.86 ± 10.92	54.94 ± 11.28
Height (cm)	144.13 ± 8.36	144.85 ± 7.86	143.13 ± 8.99
BMI (kg/m^2)	26.77 ± 3.62	26.92 ± 3.74	26.58 ± 3.47
BF (%)	44.50 ± 5.31	42.870 ± 5.04	45.65 ± 5.87
Physical activity, energy intake, and circadian variable			
Moderate-to-vigorous physical activity (min/day)	51.07 ± 19.85	58.66 ± 20.89	40.21 ± 11.72
Energy intake (Kcal/day)	1624.3 ± 430.77	1639.7 ± 433.17	1601.8 ± 431.39
Fragmentation (IV)	0.42 ± 0.05	0.41 ± 0.05	0.44 ± 0.05
Cardiorespiratory fitness			
VO2peak (ml/kg/min)	37.31 ± 4.73	37.71 ± 4.94	36.77 ± 4.14
HR and HRV			
Mean HR (bpm)	81.49 ± 9.71	80.4 ± 9.33	82.93 ± 10.12
Mean RR (ms)	746.90 ± 90.74	755.83 ± 87.3	734.60 ± 94.86
RMSSD (ms)	60.07 [56.51]	61.50 [51.06]	58.77 [49.22]
pNN50 (%)	30.94 [38.49]	33.59 [39.36]	26.87 [35.45]
SDNN (ms)	60.10 [42.44]	62.61 [47.82]	55.78 [37.09]
FFT TP (ms^2)	3068.13 [5404.60]	3678.96 [5584.59]	2611.82 [3771.87]
FFT HF (ms^2)	1222.92 [2641.93]	1410.64 [2945.46]	1142.54 [2316.98]
FFT LF (ms^2)	1287.67 [1723.94]	1331.40 [1687.08]	1260.57 [1385.01]
FFT LF/HF (ms^2)	0.92 [1.17]	0.92 [1.20]	0.91 [1.13]

Data presented as mean ± SD, and as number and frequency. Median (IQR: interquartil range) are presented for standard HRV parameters because these variables presented skewed distributions. PHV, peak height velocity; BMI, body mass index; BF%, body fat; MVPA, moderate-to-vigorous physical activity; IV, intradaily variability; HR, heart rate; RR, R-R intervals; RMSSD, the square root of the mean of the sum of the squares of differences between adjacent NN intervals; pNN50, percentage (%) of the total pairs of RR intervals that differ by more than 50 ms; SDNN, standard deviation of all normal R-R intervals; TP, total power; FFT, fast fourier transformation; HF, high frequency band index of parasympathetic activity; LF, low frequency band mix index of sympathetic and parasympathetic activity.

TABLE 2 | Pearson bivariate correlations of HR, standard and corrected HRV parameters with cardiorespiratory fitness (CRF) in overweight/obese children.

	Cardiorespiratory fitness, VO_2peak (mL/kg/min)			
	HR and standard HRV parameters		Corrected HRV parameters	
	r	p	r	p
Mean HR (beats/minute)	**−0.333***	**<0.001**	Not applicable	Not applicable
RMSSD (ms)	**0.225**	**0.020**	−0.068	0.489
pNN50 (%)	**0.268**	**0.005**	−0.070	0.475
SDNN (ms)	**0.209**	**0.030**	−0.067	0.492
FFT TP (ms^2)	0.157	0.107	−0.146	0.134
FFT HF (ms^2)	0.174	0.073	−0.043	0.658
FFT LF (ms^2)	0.080	0.415	−0.026	0.794
FFT LF/HF (ms^2)	−0.164	0.092	−0.120	0.219

$p < 0.001$. Bold numbers indicate $p < 0.05$.

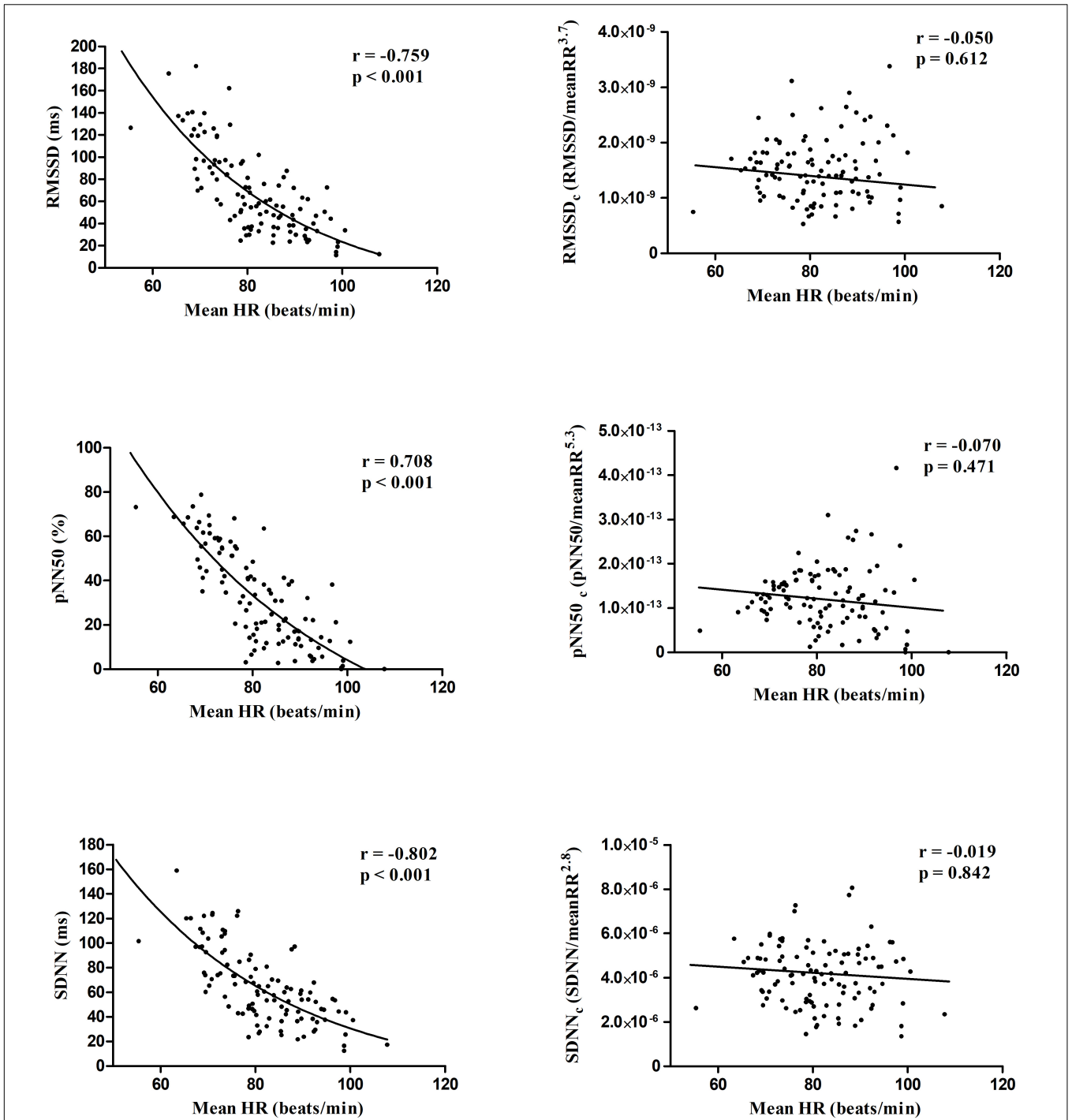

FIGURE 1 | Scatter plots showing the Spearman correlations of time domain standard (left panels) and corrected (right panels) HRV parameters with mean heart rate.

In this context, it has been reported that HRV was significantly associated with CRF independently of age, race, sex and adiposity in adolescents (Gutin et al., 2005). However, in our study we show that the associations between HRV and CRF was not significant after controlling for potential confounders (sex, maturation, adiposity, moderate-to-vigorous physical activity, energy intake, circadian-related variable and HR). These contradictory findings can be explained by different factors: participants of different ranges of age (adolescents 14–18 years vs. overweight/obese children 8–11 years); use of different HRV protocol and instrument (ECG vs. HR monitor) and different methodology of CRF assessment (VO_2 at HR of 170 bpm vs. maximal incremental

TABLE 3 | Stepwise linear regressions of HR, standard and corrected HRV parameters with cardiorespiratory fitness (CRF) in overweight/obese children.

Variables initially entered in the model	Significant predictors ($p \leq 0.05$)	*p*-value	Standardized beta coefficient	Coefficient of determination R^2
HR and standard HRV parameters: RMSSD, pNN50, SDNN, FFT TP, FFT HF, FFT LF, FFT LF/HF	HR	<0.001	−0.333	0.111
HR and corrected HRV parameters: $RMSSD_c$, $pNN50_c$, $SDNN_c$, FFT TP_c, FFT HF_c, FFT LF_c, FFT LF/HF_c	HR	<0.001	−0.333	0.111

treadmill test using a gas analyzer). Also, lack of studies in pediatric population studying associations between HRV and CRF should be considered (Oliveira et al., 2017), specifically in overweight/obese children.

To date, the majority of studies have often omitted the dependence of HRV on HR in overweight/obese children and adolescents (Rodríguez-Colón et al., 2011; Baum et al., 2013; Da Silva et al., 2014; Redón et al., 2016). This dependency might have significant influence on HRV since resting HR is different per each individual. In this sense, a correction procedure has been proposed which is based on ratios between the HRV parameters and different powers of their corresponding mean R-R intervals (Sacha et al., 2013b). In fact, the correction of HRV for prevailing HR is a kind of adjustment for HR at the individual level and it provides HRV parameters which are independent on HR. This method allows us to investigate the HR impact on the prognostic power of HRV in various clinical circumstances, e.g., this impact turned out to be positive for the prediction of cardiac death but negative for the prediction of non-cardiac death among patients after myocardial infarction (Sacha et al., 2013a). Moreover, in women, who usually present high HR, the exclusion of the HR impact on HRV improved the HRV prediction capability for all modes of death (Sacha et al., 2014). However, only one study has used the corrected parameters in relation to CRF in young adults and found that HR was a better predictor of CRF than the standard and corrected HRV parameters (Grant et al., 2013). In addition, they found that from all HRV indices, pNN50 was the most strongly associated with CRF, but HR was a better indicator of CRF than all the standard and corrected HRV parameters in young adults (Grant et al., 2013).

Our results in overweight/obese children are in line with the previous study in young adults (Grant et al., 2013). We found that some standard HRV parameters were associated with CRF, yet they lost this association after correction for HR, i.e., none of the corresponding corrected HRV parameter correlated with CRF (**Table 2**) – thus, HR seems to be a driving force in the association between standard HRV and CRF. The multiple regression analysis also confirmed that HR, but not HRV, is the only independent indicator of CRF. However, comparing with the study by Grant et al., our multiple stepwise regression revealed that only HR was a significant determinant (explained 11.1% of changes on CRF), but Grant et al. showed that both HR and HF were relevant (explained 17 and 3.1% respectively). Beyond the age range and weight status, the only methodological remarkable difference between our study and the previous one was that we measured CRF directly from gas analyzer (i.e., lab test) and Grant et al. (Grant et al., 2013) estimated CRF from the Cooper 12 min test (i.e., field test). Collectively, previous findings in young adults and present findings in overweight/obese children suggest that the associations between HRV and CRF exit mainly due to relationship between HR and CRF.

To our knowledge this is the first study showing that HR, as indicator of cardiac autonomic function, is a stronger predictor of CRF than HRV parameters in time and frequency domain in overweight/obese children. Therefore, it seems that the more complex concept of HRV do not provide additional information compared with the HR measurement in children with weight disturbances. This may have an important practical consequence, i.e., therapeutic interventions in such children should be directed at the reduction of HR which is an easy parameter and provides valuable information on CRF. However, future studies should perform these analyses in other populations of different age ranges (i.e., older adults) and health status (i.e., healthy children, obese adults, older adults with cardiovascular diseases, etc.).

Strengths and Limitations

Several limitations need to be acknowledged: (1) since our study design is cross-sectional, we cannot assume a causal relationship; (2) we did not use a gold standard for the HRV measurement, however, the RS800CX has demonstrated to be valid (Gamelin et al., 2008) and reliable (Vasconcellos et al., 2015) for this measurement; (3) some studies have found HRV parameters to be affected by breathing (Wessel et al., 2009; Sidorenko et al., 2016), but this effect is not clear and some parameters can be less affected by changes in respiration (Hill and Siebenbrock, 2009), so we decided not to control the breathing to avoid disturbing the resting status of the participants; (4) HRV analyses were performed only in time and frequency domain, maybe other complex approaches to analyze HRV (i.e., approximate and sample entropies, detrended fluctuation analyses short term exponent, etc.,) (Sassi et al., 2015) could add additional information in relation to the prediction of CRF. The main strengths of our study were: (1) CRF was objectively measured with a gas analyzer (gold standard), using an adapted protocol recommended to low-fit children and (2) to the best of our knowledge this is the first study confirming that HR is

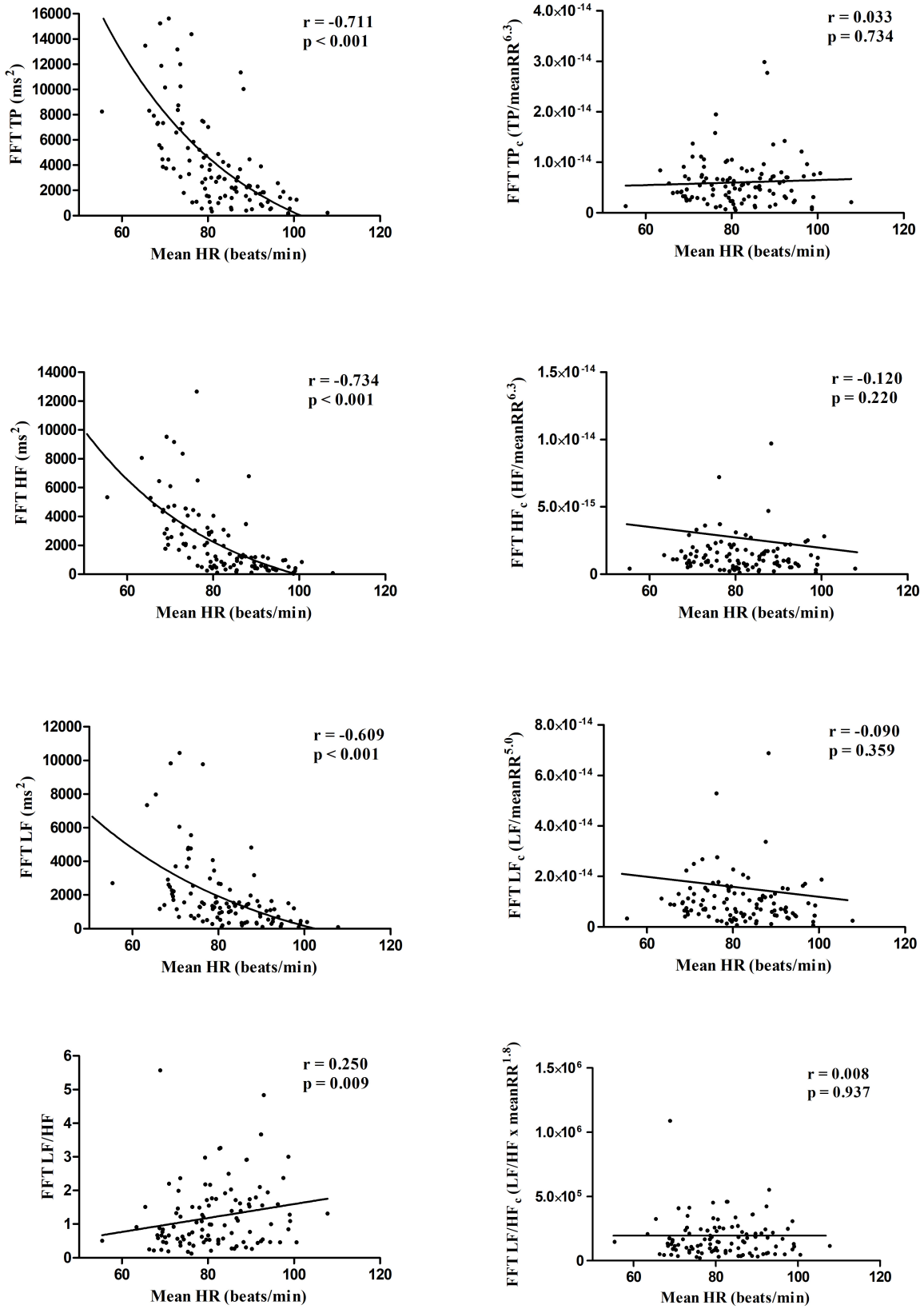

FIGURE 2 | Scatter plots showing the Spearman correlations of frequency domain standard (left panels) and corrected (right panels) HRV parameters with mean heart rate.

a better predictor of CRF than complex HRV parameters in overweight/obese children.

CONCLUSION

In conclusion, HR is a better predictor of CRF than HRV parameters, in either time or frequency domains, in overweight/obese children. Further, HRV parameters did not add any significant information to the models in our overweight/obese participants. Thus, the complex concept of HRV might not provide additional information to the prediction of CRF. Clinicians and sport scientists that use cardiac autonomic activity as indicator of cardiovascular health represented by CRF should take into consideration that the "simple" HR metric gives more information about CRF levels of overweight/obese children than any HRV parameter. Furthermore, the HR assessment and its interpretation are markedly easier than the ones of HRV parameters.

ETHICS STATEMENT

This study was conducted according to the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014).

AUTHOR CONTRIBUTIONS

JM, J M-G, CC-S, and FO contributed to design the experiment and study design. AP-F, JM, JM-G, PM-G, MR-A, CC-S, IE-C, JS, and FO contributed to acquisition, analysis, or interpretation of data for the manuscript. All authors drafted or critically revised the manuscript for important intellectual content and approved the final version of the manuscript to be published.

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SUPPLEMENTARY MATERIAL

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frontiers in Physiology

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The Role of Heart Rate on the Associations Between Body Composition and Heart Rate Variability in Children With Overweight/Obesity: The ActiveBrains Project

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Background: Heart rate variability (HRV) is negatively associated with body mass index and adiposity in several populations. However, less information is available about this association in children with overweight and obesity, especially severe/morbid obesity, taking into consideration the dependence of HRV on heart rate (HR).

Objectives: (1) to examine associations between body composition measures and HRV, (2) to study differences in HRV between children with overweight and severe/morbid obesity; and (3) to test whether relationships and differences tested in objectives 1 and 2, respectively are explained by the dependency of HRV on HR.

Methods: A total of 107 children with overweight/obesity (58% boys, 10.03 ± 1.13 years) participated in this study. Body composition measures were evaluated by Dual-energy X-ray absorptiometry (DXA). HRV parameters were measured with Polar RS800CX®.

Results: Body composition measures were negatively associated with HRV indicators of parasympathetic activity (β values ranging from −0.207 to −0.307, all $p < 0.05$). Children with severe/morbid obesity presented lower HRV values with respect to children with overweight/mild obesity in HRV parameters indicators of parasympathetic

activity (p = 0.035). All associations disappeared after further adjustment for HR (all $p > 0.05$).

Conclusion: All associations between adiposity/obesity and HRV could be explained by HR, suggesting a key confounding role of HR in HRV studies in children with weight disturbances.

Keywords: parasympathetic, sympathetic, fat mass, adiposity, preadolescents

INTRODUCTION

Childhood obesity is one of the major public health concerns worldwide (World Health Organization [WHO], 2017). Evidence points out that pediatric obesity, especially severe obesity, is a risk factor for the development of insulin resistance, low high-density lipoprotein cholesterol levels and high systolic blood pressure, among others (Wells et al., 2004; Skinner et al., 2015). Low parasympathetic activity (PA) has been also proposed as an important risk factor for cardiovascular disease and mortality (Thayer and Lane, 2007; Thayer et al., 2010) and related to several body composition (BC) measures (Gutin et al., 2000; Nagai et al., 2003; Rodríguez-Colón et al., 2011). PA can be assessed by measuring heart rate variability (HRV) which refers to the variability in the time interval between consecutive heart beats (variability of the R−R interval) (Task Force, 1996), so that lower HRV values indicate a lower PA.

A negative association has been reported between several BC measures [i.e., fat mass and fat-free mass (Gutin et al., 2000), body weight, and waist circumference (WC) (Rodríguez-Colón et al., 2011), and body mass index (BMI) (Birch et al., 2012)] with HRV parameters in time- and frequency-domain (hereinafter referred to as HRV parameters) either in children with normal weight (NW), overweight (OW), or obesity (OB). Accordingly, some studies found lower HRV values in children with OB compared to their peers with NW and OW (Martini et al., 2001; Nagai et al., 2003; Rabbia et al., 2003; Kaufman et al., 2007; Rodríguez-Colón et al., 2011). Conversely, Redón et al. (2016) did not find significant differences in HRV across different weight status groups (i.e., children with OW, mild OB and severe OB). These controversies can be explained by methodological differences between studies (i.e., time of recording HRV, the instrument employed to record HRV, children with different weight status). Otherwise, to the best of our knowledge, the inclusion of subgroups of children with severe/morbid OB is currently lacking with only one study including children with severe obesity (Redón et al., 2016). Importantly, HRV strongly depends on heart rate (HR) (Sacha and Pluta, 2008; Sacha, 2013, 2014b). This dependency has been shown to be an important factor to consider when analyzing HRV associations with health outcomes (Trimmel et al., 2015), but previous studies have failed in considering this dependency. This is particularly important in this population because those often have higher HR, and thus there is a higher chance of HRV underestimation.

Therefore, the aims of this study were: (1) to examine associations between BC measures (i.e., BMI, WC, WC/height, fat mass index, body fat percentage, fat free mass index, and visceral adipose tissue) and HRV parameters in time and frequency-domain, (2) to study differences in HRV across children with different weight status, and (3) to test whether the relationships between BC measures and HRV are explained by the dependency of HRV on HR in children with OW/OB.

MATERIALS AND METHODS

Study Design and Participants

This cross-sectional study used baseline data of 107 children with OW/OB (9–11 years, 58% boys) from the ActiveBrains project[1] (Cadenas-Sánchez et al., 2016). The age group (9–11 years) was made on a preadolescent sample due to adolescent physiological and psychological changes are dramatic, and it is difficult to control confounding factors. Detailed design and methods have been described elsewhere (Cadenas-Sánchez et al., 2016). For feasibility reasons, the ActiveBrains project was conducted in three waves, carrying out the baseline assessments from October 2014 to February 2016. This study was conducted according to the Declaration of Helsinki. The protocol was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). All parents had received information about the study and gave written informed consent in accordance with the Declaration of Helsinki.

Body Composition Measures

Body weight and height were measured with an electronic scale and a stadiometer (Seca instruments, Germany, Ltd.). BMI was calculated as kg/m^2 and participants were then classified as children with OW, mild, severe or morbid OB according to the sex- and age-specific international BMI standards (World Obesity Federation) (Cole and Lobstein, 2012; Bervoets and Massa, 2014). We combined children with severe and morbid OB due to their low prevalence in our sample (i.e., severe OB: n = 22, 20.6%; morbid OB: n = 11, 10.3%). WC was evaluated as an indicator of central fat using the International Society for the Advancement of Kinanthropometry (ISAK) procedures (ISAK, 2006). Body weight, height, and WC were collected twice consecutively by the same trained evaluator, and the average for each parameter was used. Fat mas and fat-free mass were measured by dual energy X-ray absorptiometry (DXA, Discovery densitometer from Hologic). Fat mass index, fat-free mass index and body fat percentage (FMI, FFMI, and BFP, respectively) were

[1]http://profith.ugr.es/activebrains?lang=en (Clinical Trial: NCT02295072).

derived as the ratio between fat mass and fat-free mass (kg) with the squared height (m^2), and the percentage (%) of adipose tissue relative to body weight, respectively. Also, visceral adipose tissue (VAT) was measured with DXA.

Heart Rate Variability

Participants were placed supine for 10 min in a quiet and comfortable room between 9 a.m. and 12 p.m. Supine position has shown a higher reliability for the HRV measurement in children than sitting or standing (Silva et al., 2016). The POLAR RS800CX (Polar Electro Oy Inc., Kempele, Finland) recorded HRV during 10 min at a sampling frequency of 1000 Hz. This HR monitor provides valid measures with respect to ECG (Gamelin et al., 2008) and it is reliable for the HRV assessment in children and adolescents (Vasconcellos et al., 2015). Participants were encouraged to breathe normal, keep relaxed and to not move or speak during the evaluation.

We used the normal R–R intervals after excluding the extreme values with the automatized low filter available in the Kubios software (HRV analysis, University of Eastern Finland) (Niskanen et al., 2004; Tarvainen et al., 2014). The R–R interval series were detrended using the smoothness prior method with alpha set at 500 and a cubic interpolation at the default rate of 4 Hz. Out of the 10 min recorded, the middle 5 min (i.e., from minutes 3 to 8) were checked for quality (i.e., normal distribution of the R–R intervals, no large R–R interval outliers and R–R intervals equidistance and minimal variation) and a different period of 5-min was selected when necessary. We derived time- and frequency-domain HRV parameters based on the Guidelines of Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology (Task Force, 1996). In the time-domain, we computed the squared root of the mean of the sum of the squares of successive normal R–R interval differences (RMSSD) and the percentage number of pairs of adjacent normal R–R intervals differing by more than 50 ms in the entire recording (pNN50) as indexes of PA. We also computed the standard deviation of all normal R–R intervals (SDNN). In the frequency-domain, we performed spectral analyses using the non-parametric fast Fourier transformation algorithm (FFT), with Welch's periodogram method (i.e., 50% overlap Hanning window as pre-processing technique and calculating area under the curve with an integration). We derived total power (TP) of HRV spectrum, the power in the high (HF) and the low frequency (LF) bands (TP: 0–0.4 Hz; LF: 0.04–0.15 Hz; HF: 0.15–0.4 Hz) in absolute units (ms^2). HF and the LF/HF ratio were then used in the analyses as indicators of PA and sympatho-vagal balance, respectively (Task Force, 1996), although physiological significance of LF/HF ratio is not clear (Goldstein et al., 2011; Billman, 2013).

To remove the HRV dependence on HR, we calculated the corrected HRV parameters proposed by Sacha et al. (2013) based on two assumptions: (1) If HRV parameters were negatively correlated with HR, the correction procedure consisted in calculating ratios between HRV parameters and different powers of their corresponding mean normal R–R interval; (2) if HRV parameters were positively correlated with HR, the correction procedure was performed by multiplying HRV parameters by the adequate powers of mean normal R–R interval as follows: $RMMSDc = RMSSD/meanRR^{3.7}$, $pNN50c = pNN50/meanRR^{5.3}$, $SDNNc = SDNN/meanRR^{2.8}$, $FFT\ TPc = TP/meanRR^{6.3}$, $FFT\ HFc = HF/meanRR^{6.3}$, $FFT\ LFc = LF/meanRR^{5.0}$, $FFT\ LF/HFc = LF/HF \times meanRR^{1.8}$.

Basic Confounders

Peak height velocity (PHV) was derived from measured height and seated height as a discriminant measure of maturational status (Malina et al., 2015; Moore et al., 2015). Maturity offset was calculated by subtracting the PHV age from the chronological age and used in the analyses.

Socioeconomic status was assessed by parental education level. Both mother and father self-reported their highest educational level as no elementary school, elementary school, middle school, high school, and university degree completed. Parent responses were then combined as follows: none of the parents had a university degree, one of the parents had a university degree or both parents had a university degree.

Since each wave of participants was evaluated in different months of the year, and it could affect to HRV, we decided to add wave as a dummy covariate in our models to control for the potential seasonal variance in the outcomes.

Statistical Analyses

Descriptive characteristics are presented as means and standard deviations (mean ± SD) for continuous variables that exhibited a normal distribution, medians, and interquartile ranges for continuous non-normal variables, and frequencies, and percentages for categorical variables. Values of corrected HRV parameters were presented as medians with the 5th and 95th percentiles in the same way that were presented previously by Gąsior et al. (2018) in healthy weight children. Kolmogorov–Smirnov test and a visual inspection of histograms were performed to assess the normal distribution of variables. Normal scores of HRV parameters were calculated according to the Blom formula (Blom, 1954, 1958) to obtain normally distributed variables when needed.

Spearman correlation tests were performed to study associations between standard (without correction by HR) and corrected HRV parameters with mean HR to confirm their dependence and their loss of dependency after performing the Sacha et al. (2013) correction. Sex, PHV offset, parent education university level, and wave (transformed into binary variables) were selected as basic confounders. Stepwise linear regression models were performed to test the relevance of basic confounders in all models, only variables included in the stepwise models were carried forward to the final model with BC measures as predictors and HRV parameters as outcomes. Associations of BC measures with HRV parameters were studied using linear regressions. Model 1 included only the selected basic confounders by the stepwise models, and standard HRV parameters as outcomes. Model 2 included the same confounders than model 1 plus mean HR, and standard HRV as outcomes (Van De Wielle and Michels, 2017). Lastly, model 3 included the selected basic confounders by the stepwise models, and corrected HRV parameters as outcomes (Sacha et al., 2013).

Finally, we used analysis of covariance (ANCOVA) to explore differences in HRV parameters across weight status groups (i.e., OW, mild, severe/morbid OB). ANCOVA models were adjusted for the same confounders introduced in linear regression models 1 and 2. We only performed ANCOVA adjusted by HR (model 2) and did not show this analysis with corrected HRV parameters (HRV_c) (model 3) as the results were the same independently of the method performed to remove the dependence of HRV on HR. Statistical significance was defined at the level of $p < 0.05$ for all the analyses. Analyses were performed using SPSS version 21.0 (IBM Corporation, NY, United States).

RESULTS

Table 1 shows the descriptive characteristics of participants stratified by weight status (i.e., OW, mild, severe/morbid OB groups). As expected, BC measures showed significant differences between weight status groups (all $p \leq 0.001$). Also, children with severe/morbid OB presented significantly higher mean HR than their peers with OW and mild OB ($p < 0.002$ and $p < 0.001$, respectively). For descriptive purposes, **Table 2** compares corrected HRV parameters analyzed in our study with normative corrected HRV parameters recently published in healthy weight children (Gąsior et al., 2018). It can be observed that our sample of children with OW/OB have lower values of HRV than healthy weight children.

Supplementary Figures S1, S2 present the Spearman correlations between HRV parameters and HR. Significant correlations found between the standard HRV parameters and HR (r ranging from −0.802 to 0.250; all $p \leq 0.009$) disappeared after the correction procedure (r ranging from −0.120 to 0.033; $p \geq 0.220$).

Table 3 shows associations between BC measures and HRV parameters. Concerning to time-domain HRV parameters, the model 1 (basic confounders tested were sex, PHV offset, parent education university level and wave but none were included after performing stepwise analyses) showed negative associations of BMI with RMSSD and pNN50 ($\beta = -0.207$ and $\beta = -0.240$, respectively; both $P < 0.032$). Also, WC/height ratio was negatively associated with pNN50 ($\beta = -0.194$, $p = 0.046$). FMI and BFP from DXA were negatively associated with RMSSD, pNN50 and SDNN (β ranging from −0.203 to −0.272; p ranging from 0.005 to 0.036). In regard to HRV parameters in frequency-domain, BMI and FMI were negatively associated with HF ($\beta = -0.214$ and $\beta = -0.208$, respectively; both $P < 0.032$). Also, BMI, WC, FMI, and FFMI were positively associated with LF/HF (β ranging from 0.201 to 0.283; both p ranging from 0.002 to 0.038). In model 2 (same confounders as model 1 plus mean HR) and model 3 (selected basic confounders by the stepwise models in model 1, and corrected HRV parameters as outcomes), the previously found associations between BC measures and mainly HRV indicators of PA (RMSSD, pNN50, and HF) in time- and frequency-domain were no longer significant (all $p > 0.05$).

Figure 1 depicts differences on standard HRV parameters across weight status groups in model 1 and model 2. Concerning HRV parameters in time-domain, there were (model 1) no significant differences for RMSSD ($p = 0.056$) and SDNN ($p = 0.299$), but significant differences across weight status for pNN50 ($p = 0.023$). Bonferroni *post hoc* analysis did not show significant differences for pNN50 across weight status groups.

Regarding frequency-domain HRV parameters, no significant differences were reported for LF ($p = 0.557$) and TP ($p = 0.380$) across weight status groups. Importantly, children with severe/morbid OB showed significantly lower values on HF in respect to peers with mild OB ($p = 0.036$) and borderline differences in LF/HF across weight status ($p = 0.061$). All these differences disappeared after additional adjustment for mean HR (model 2, all $p > 0.05$).

DISCUSSION

Main Findings

The main findings of this study were: BC measures (BMI, WC/Height, FMI, and BFP) were negatively associated with HRV indicators of PA (RMSSD, pNN50, and HF) and children with severe/morbid OB had lower values of HRV (RMSSD, pNN50, and HF) compared to their peers with OW and mild OB. However, nearly all associations of BC measures with HRV parameters, as well as differences across weight status groups, were fully explained by HR.

Associations Between BC Measures and HRV Parameters

Some HRV parameters in time and frequency-domain are considered indicators of PA (i.e., RMSSD, pNN50, and HF). For time-domain parameters, we found inverse associations of BMI with RMSSD and pNN50. Also, FMI and BFP were negatively associated with RMSSD, pNN50 and SDNN. Accordingly, Gutin et al. (2000) found negative significant associations of fat mass and fat-free mass (from DXA) with RMSSD. Likewise, we did not find associations between VAT and HRV in our sample, in accordance with this previous study (Gutin et al., 2000). In contrast to Gutin et al. (2000), we did not find associations between FFMI and RMSSD. HRV methodological differences, such as different instrument (i.e., ECG vs. HR monitor), differences in sample characteristics (i.e., children with obesity vs. children with overweight/obesity in our study) and different confounders could be responsible for these inconsistent findings between studies.

Furthermore, in relation to HRV parameters in frequency-domain we found negative associations of BMI, and FMI, with HF used as indicator of PA. Also, we found positive associations of BMI, WC, FMI, and FFMI with LF/HF. In the same line, previous studies found similar results in children with OW/OB (Baum et al., 2013; Santos-Magalhaes et al., 2015). For example, Baum et al. (2013) found negative and positive associations of BMI with HF and LF/HF ratio, respectively. Santos-Magalhaes et al. (2015), similar to our study, showed significant positive associations between WC, as indicator of central fat, and LF/HF ratio. Some authors have considered LF/HF ratio as an indicator of sympatho-vagal balance (Pagani et al., 1986; Malliani et al., 1991). Under this assumption, our

TABLE 1 | Descriptive characteristics of participants.

Variables	Total sample (*n* = 107)	Overweight (*n* = 28, 26%)	Mild-obesity (*n* = 46, 43%)	Severe/Morbid-obesity (*n* = 33, 31%)	*P* value
Boys, *n* (%)	62 (58)	16 (57)	29 (63)	17 (52)	0.592
Parents with university degree, *n* (%)					0.005**
None of them	71 (66)	15 (54)	27 (59)	29 (88)[a,b]	
One of them	19 (18)	6 (21)	10 (22)	3 (9)[a,b]	
Both of them	17 (16)	7 (25)	9 (20)	1 (3)[a,b]	
Wave, *n* (%)					0.905
Wave 1	19 (18)	5 (18)	8 (17)	6 (18)	
Wave 2	43 (40)	10 (36)	20 (44)	13 (39)	
Wave 3	45 (42)	13 (46)	18 (39)	14 (42)	
Age (years)	10 ± 1	10 ± 1	10 ± 1	9 ± 1[a,b]	0.004**
PHV offset (years)	−2.26 ± 0.99	−2.25 ± 1.01	−2.06 ± 1.02	−2.54 ± 0.90	0.107
Body composition					
Weight (kg)	56 ± 11	46 ± 7	58 ± 10[a]	62 ± 9[a]	< 0.001**
Height (cm)	144 ± 8	142 ± 9	147 ± 8[a]	142 ± 7[b]	0.014**
BMI (kg/m^2)	27 ± 3	22 ± 1	26 ± 2[a]	31 ± 2[a,b]	< 0.001**
WC (cm)	90 ± 10	80 ± 6	90 ± 8[a]	98 ± 7[a,b]	< 0.001**
WC/Height	0.62 ± 0.06	0.57 ± 0.03	0.62 ± 0.04[a]	0.69 ± 0.04[a,b]	< 0.001**
DXA FM (Kg)	25 ± 7	18 ± 4	25 ± 5[a]	30 ± 7[a,b]	< 0.001**
DXA FMI (kg/m^2)	12 ± 3	9 ± 1	11 ± 2[a]	15 ± 2[a,b]	< 0.001**
DXA BFP (%)	44 ± 6	39 ± 4	43 ± 4[a]	49 ± 4[a,b]	< 0.001**
DXA FFM (Kg)	31 ± 5	28 ± 5	32 ± 6[a]	31 ± 4[a]	< 0.001**
DXA FFMI (kg/m^2)	15 ± 1	14 ± 1	15 ± 1[a]	15 ± 1[a]	< 0.001**
DXA Total VAT (g)*	398 ± 115	297 ± 73	397 ± 97[a]	484 ± 99[a,b]	< 0.001**
DXA VAT FMI (g)*	191 ± 51	146 ± 33	182 ± 34[a]	240 ± 41[a,b]	< 0.001**
Heart rate variability					
Mean HR (bpm)	81 ± 10	79 ± 10	79 ± 9	87 ± 8[a,b]	< 0.001**
Mean RR (ms)	747 ± 91	774 ± 101	767 ± 87	695 ± 63[a,b]	< 0.001**
RMSSD (ms)	60 [57]	71 [55]	72 [83]	51 [28]	0.056
pNN50 (%)	31 [38]	41 [38]	37 [43]	21 [19]	0.023**
SDNN (ms)	60 [42]	68 [44]	65 [54]	54 [25]	0.299
FFT TP (ms^2)	3068 [5405]	3736 [5609]	4084 [5997]	2349 [2339][a]	0.035**
FFT HF (ms^2)	1223 [2642]	1987 [3212]	2140 [3731]	860 [814]	0.380
FFT LF (ms^2)	1288 [1724]	1291 [2023]	1409 [1903]	1151 [1195]	0.577
FFT LF/HF	0.9 [1.2]	0.8 [1.2]	0.8 [1.0]	1.5 [1.5]	0.061

*Data presented as mean ± SD, as number and frequency. Median [IQR: Interquartile range] are presented for standard HRV parameters because these variables presented skewed distributions. PHV, peak height velocity; BMI, body mass index; WC, waist circumference; DXA, dual energy X-ray absorptiometry; FM: fat mass; FMI, fat mass index; BFP, body fat percentage; FFM, fat-free mass; FFMI, fat-free mass index; VAT, visceral adipose tissue; HR, heart rate; RR, R–R intervals; RMSSD, The square root of the mean of the sum of the squares of differences between adjacent R–R intervals; pNN50: percentage (%) of the total pairs of R–R intervals that differ by more than 50 ms; SDNN, standard deviation of all normal R–R intervals; FFT, fast fourier transformation; HF, high frequency band (0.15–0.4 Hz) index of parasympathetic activity; LF, low frequency band (0.04–0.15 Hz) mix index of sympathetic and parasympathetic activity. *Indicates that these variables have some missing values, i.e., DXA Total VAT (g): n = 19; DXA VAT FMI (g): n = 19; P value column denotes significant differences among groups. [a]Denotes significant differences ($p < 0.05$) respect to the subgroup of children with overweight. [b]Denotes significant differences ($p < 0.05$) respect to the subgroup of children with mild obesity. **$p < 0.05$.*

findings suggest that higher BMI, WC and fat mass levels are associated with higher predominance of sympathetic activity over PA in children with overweight/obesity. However, physiological significance of LF/HF ratio is not clear in the literature (Goldstein et al., 2011; Billman, 2013). For example, has been reported that beta-adrenergic receptor blockade combined with parasympathetic denervation supposed an increase of LF/HF ratio from 1.1 to 8.4, which suggest a "false" sympathetic dominance (Randall et al., 1991; Billman, 2013). Importantly, some interventions such as myocardial ischemia or exercise did not increase LF, instead provoked a significant reduction in LF power (Houle and Billman, 1999). The interpretation of the LF/HF ratio findings as indicator of sympatho-vagal balance should be considered with caution [see Billaman article for revision (Billman, 2013)].

HRV Differences Between Weight Status Groups

Children with severe/morbid OB presented lower RMSSD, pNN50 and HF (indicators of PA) and higher LF/HF ratio compared to their peers with OW and mild OB in our study. These differences were borderline significant for RMSSD and

TABLE 2 | Normative values of corrected HRV parameters in children with overweight/obesity and values published previously in healthy weight children (Gąsior et al., 2018).

	Overweight/obesity (*n* = 107)			Healthy children (*n* = 312)		
HRV PARAMETER	**Median**	**5th percentile**	**95th percentile**	**Median**	**5th percentile**	**95th percentile**
$RMSSD_c$	1.4E-09	7.1E-10	2.6E-09	1.4E-07	7.6E-08	2.6E-07
$pNN50_c$	1.6E-14	2.0E-15	3.6E-14	1.4E-13	2.0E-14	3.0E-13
$SDNN_c$	5.6E-07	2.6E-07	8.7E-07	2.6E-05	1.5E-05	4.6E-05
FFT TP_c	3.0E-15	1.0E-15	7.0E-15	1.2E-11	3.6E-12	3.8E-11
FFT HF_c	1.1E-15	3.0E-16	3.3E-15	6.8E-12	1.7E-12	2.5E-11
FFT LF_c	5.2E-12	1.1E-12	1.8E-11	2.9E-09	7.5E-10	9.8E-09
FFT LF/HF	1.4E+05	3.5E+04	4.4E+05	4.5E+02	1.5E+02	1.2E+03

Corrected parameters were calculated as follows in our sample: RMMSDc = RMSSD/meanRR$^{3.7}$, pNN50c = pNN50/meanRR$^{5.3}$, SDNNc = SDNN/meanRR$^{2.8}$, FFT TPc = TP/meanRR$^{6.3}$, FFT HFc = HF/meanRR$^{6.3}$, FFT LFc = LF/meanRR$^{5.0}$, FFT LF/HFc = LF/HF x meanRR$^{1.8}$.

TABLE 3 | Standardized beta coefficients from linear regression models on the associations of body composition measures with standard and corrected HRV parameters.

	RMSSD	pNN50	SDNN	FFT TP	FFT HF	FFT LF	FFT LF/HF
Model 1: Standard HRV parameters; Confounders†: none							
BMI (kg/m^2)	−**0.207**	−**0.240**	−0.158	−0.117	−**0.214**	−0.018	**0.283***
WC (cm)	−0.120	−0.148	−0.052	−0.042	−0.125	0.024	**0.201**
WC/Height	−0.152	−**0.194**	−0.076	−0.065	−0.146	−0.031	0.159
DXA FMI (kg/m^2)	−**0.232**	−**0.272***	−**0.203**	−0.138	−**0.208**	−0.042	**0.250***
DXA BFP (%)	−**0.221**	−**0.261***	−**0.220**	−0.148	−0.168	−0.084	0.146
DXA FFMI (kg/m^2)	−0.062	−0.067	−0.005	−0.019	−0.122	0.048	**0.225**
DXA Total VAT (g)	−0.048	−0.090	−0.019	−0.005	−0.054	0.069	0.121
DXA VAT FMI (g)	−0.090	−0.134	−0.062	−0.036	−0.095	0.019	0.082
Model 2: Standard HRV parameters; Confounders†: HR							
BMI (kg/m^2)	−0.034	−0.058	0.003	0.046	−0.052	0.131	**0.242**
WC (cm)	−0.003	−0.026	0.057	0.067	−0.015	0.122	0.168
WC/Height	0.095	0.063	0.158	0.169	0.086	**0.179**	0.094
DXA FMI (kg/m^2)	−0.028	−0.059	−0.015	0.055	−0.015	0.135	**0.201**
DXA BFP (%)	−0.008	−0.039	−0.026	0.051	0.035	0.097	0.086
DXA FFMI (kg/m^2)	−0.039	−0.042	0.017	0.003	−0.099	0.068	**0.218**
DXA Total VAT (g)	0.050	0.013	0.075	0.100	0.043	0.132	0.092
DXA VAT FMI (g)	0.111	0.074	0.131	0.159	0.103	0.159	0.023
Model 3: Corrected HRV parameters; Confounders†: years PHV offset							
BMI (kg/m^2)	0.026	−0.042	0.010	0.109	−0.014	0.140	**0.198**
WC (cm)	0.107	−0.006	0.101	0.156	0.065	0.129	0.112
WC/Height	0.108	0.004	0.114	0.179	0.069	0.168	0.114
DXA FMI (kg/m^2)	0.012	−0.058	−0.018	0.117	0.016	0.136	0.163
DXA BFP (%)	0.012	−0.055	−0.036	0.105	0.058	0.085	0.061
DXA FFMI (kg/m^2)	0.024	−0.011	0.040	0.033	−0.073	0.090	0.183
DXA Total VAT (g)	0.102	−0.020	0.062	0.121	0.058	0.117	0.089
DXA VAT FMI (g)	0.157	0.057	0.109	0.181	0.110	0.110	0.020

*BMI, body mass index; WC, waist circumference; DXA, dual energy X-ray absorptiometry; FMI, fat mass index; BFP, body fat percentage, FFMI, fat-free mass index; VAT, visceral adipose tissue; PHV, peak height velocity. See full name of all the heart rate variability in time and frequency-domain (units in ms and ms^2, respectively) parameters in **Table 1**. †Confounders on the model were selected by stepwise regression analyses (i.e., all basic confounders considered in the three models were sex, PHV offset, parental education and wave). Additionally, mean HR was introduced as a confounder in the model 2. Stepwise analyses for confounders did not introduced any of the confounders tested in model 1, only mean HR in model 2 and PHV offset in model 3. Bold numbers indicate $p < 0.05$. *Indicates $p < 0.01$.*

LF/HF ratio, which can be assumed as lack of statistical power due to our relatively small simple size (N = 107). Vanderlei et al. (2010) found significantly lower RMSSD, pNN50, and HF values in children with OB compared to NW children. Likewise, Birch et al. (2012) found lower HF values in children with OW/OB compared to their peers with NW. Accordingly, Rodríguez-Colón et al. (2011) reported lower values in SDNN and LF in children with OB compared to children with NW

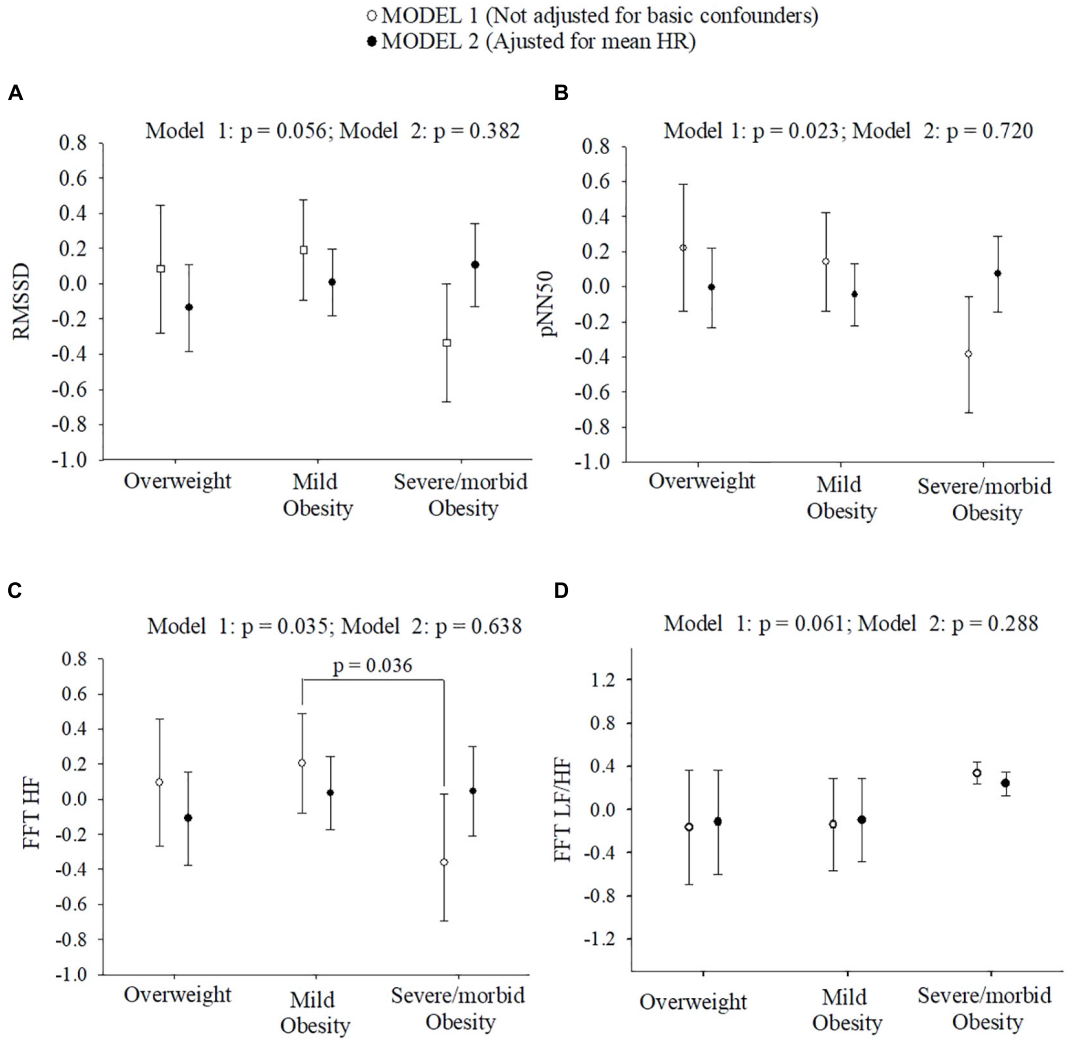

FIGURE 1 | Normal scores of HRV parameters in time and frequency-domain across children with overweight/obesity. **(A,B)** show differences of HRV parameters in time-domain across children with overweight/obesity. **(C,D)** show differences of HRV parameters in frequency-domain across children with overweight/obesity. Adjusted means and 95% confidence intervals are presented. Bonferroni *post hoc* analysis did not show significant differences for pNN50 across children with different weight status. See full name of all the heart rate variability parameters in **Table 1**.

and OW. Also, in that study, higher LF/HF ratio and HR values were reported in children with OB compared with their NW and OW peers. Otherwise, Redón et al. (2016) showed similar SDNN values across children with OW, mild and severe OB, which concurs with present findings. These differences might be explained by different instruments and durations for the HRV assessment (Task Force, 1996), i.e., 9 h of continuous ECG in the Rodríguez-Colón et al. (2011) study, 15 min with ECG in the Redón et al. (2016) study, and 10 min with HR monitor in our study. Also, the differences in sample size and characteristics between studies should be considered. For example in our study and the study of Redón et al. (2016) the sample size was relatively low (n = 107 and 64, respectively) and in general we did not find significant differences on HRV parameters between weight status groups (only significant for HF in our study). However, the sample size on Rodríguez-Colón et al. (2011) study was higher (n = 616) and they found lower values in SDNN.

The Dependence of HRV on HR and Health Outcomes

This study provides values of HRV_c in children with OW/OB with the values published previously in healthy weight children (Gąsior et al., 2018). The values of HRV_c in healthy weight children were higher than in children with OW/OB, further supporting the notion of obesity associated with a worse HRV profile. Future research should test whether differences in HRV_c observed between healthy weight children and our sample of children with OW/OB are clinically relevant. On the other hand, some methodological differences between the study of Gąsior et al. (2018) and our study should be considered to compare these values: sample size was higher than ours (n = 312 and

n = 107, respectively) compromising the representativity of the results, the instrument to record HRV signal (i.e., ECG vs. HR monitor), the range of age (i.e., 6–13 and 8–11 years, respectively) and, the HF band was fixed at different frequencies (0.5 Hz vs. 0.4 Hz, respectively).

The majority of the previous studies did not consider the dependence of HRV on HR in children with OW/OB (Rodríguez-Colón et al., 2011; Baum et al., 2013; Da Silva et al., 2014; Redón et al., 2016). It is important to note that in our study nearly every association between BC measures and HRV parameters disappeared after considering mean HR, either as confounder or after calculating the HRV_c proposed by Sacha et al. (2013). Similarly, differences across weight status groups also disappeared after considering the influence of mean HR. Our study suggests that HRV differences across weight status groups are explained by differences in mean HR, independently of the method used to remove the HRV dependence on HR. It should be underlined that standard HRV actually provides information on two quantities, i.e., on HR and its variability and it is hard to determine which of these two plays a principal role in the clinical value of HRV (Sacha, 2013, 2014b). Furthermore, the association between HRV and HR is not only a physiological phenomenon but also a mathematical one, which is due to non-linear (mathematical) relationship between RR interval and HR (Sacha and Pluta, 2008). However, by removing HRV dependence on HR one explores the HR contribution to the physiological and clinical significance of HRV in a given clinical context (Sacha, 2013, 2014b).

To our knowledge, the influence of HR on the associations between BC and HRV parameters has not been previously tested in children. However, the influence of HR on the associations between other health outcomes and HRV have been reported in children with NW and young adults (Grant et al., 2013; Van De Wielle and Michels, 2017). In the context of physical fitness and health, associations between HRV and VO_2max were lost after removing the dependence of HRV on HR, so these associations are explained by the relationships between mean HR and VO_2max (Grant et al., 2013). Also, the contribution of HR to the clinical value of HRV has been reported (Sacha, 2014a,c). In fact, Sacha et al. (2013) showed that prediction capacity of HRV in relation to cardiovascular mortality worsened after removing the dependence of HRV on HR, especially for populations and events where HR was a strong risk factor (Sacha, 2014c). Similar to previous findings, our study suggests that the relationships between health parameters (i.e., VO2max, blood biomarkers, BC measures) and HRV should be considered the influence of HR. This is an important observation that should be tested in future studies with different health outcomes in children with OW/OB and other populations (i.e., older adults, older adults with severe/morbid obesity, older adults with cardiovascular diseases).

Strengths and Limitations

Several limitations need to be acknowledged in our study: (1) the cross-sectional design of this study does not allow for causal interpretation; (2) we did not use a gold standard for the HRV measurement, however, the RS800CX has demonstrated to be valid and reliable for its assessment; (3) some studies have found HRV parameters to be affected by the breathing (Wessel et al., 2009; Sidorenko et al., 2016) although it depends on the HRV parameter analyzed (Hill and Siebenbrock, 2009), so we decided to not disturb the resting status of participants and to let them breath naturally; (4) we do not include children with NW in our sample to compare HRV differences with children with OW, mild, severe and morbid OB; (5) The relatively small sample size in different subgroups based on BMI. The strengths of our study were: (1) BC was measured with the method gold-standard (DXA); (2) we tested the influence of potential confounding variables such as sex, PHV offset, socioeconomic status measured as parental education and wave; and (3) to the best of our knowledge this is the first study investigating the influence of HR on the associations between BC measures and HRV in children with OW/OB, which has demonstrated to completely affect the findings.

CONCLUSION

Our study suggests that BC measures are negatively associated with HRV parameters, indicators of PA, but the associations found seem to be explained by mean HR. Likewise, children with severe/morbid OB had lower HRV values than their OW/mild OB peers, which could be explained by the higher HR values in children with severe/morbid OB compare to peers with OW/mild OB. The "simplest" concept of HR seems to be explaining the associations between BC measures and the "complex" concept of HRV in children with OW/OB. Likewise, the HR assessment and interpretation are easier than HRV parameters. Thus, future research should investigate the dependence of HRV on HR before concluding associations or effects on HRV parameters. Clinicians should take into consideration the assessment of HR and not only the measurement of HRV alone in children with weight disturbances.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was conducted according to the Declaration of Helsinki. The protocol was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). All parents had received information about the study and gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

JM, JM-G, CC-S, and FO made substantial contributions to design the experiment and study design. AP-F, JM, JM-G, PM-G, MR-A, CC-S, IE-C, JS, and FO made substantial contributions to

acquisition, analysis, or interpretation of data for the manuscript. All authors drafted and revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Longitudinal Associations of Leptin and Adiponectin with Heart Rate Variability in Children

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For early prevention of cardiovascular disease, early detection and risk factor insights are necessary. The autonomic balance reflects cardiovascular risk and can be measured by heart rate variability (HRV). Therefore, our purpose is to examine associations between HRV and the energy-related biomarkers leptin and adiponectin in children. Participants of this study were Belgian children recruited for the longitudinal ChiBS study (year 2010–2012). HRV was measured and fasting blood samples were taken in 249 children at baseline (4.4–11.0 y) and 223 children at follow-up (6.7–12.2 y). Cross-sectional and longitudinal linear regression analyses were separated by sex and adjusted for age, socio-economic status, body fat%, negative emotions, puberty, and mean heart rate. Leptin was a negative cross-sectional and longitudinal predictor of parasympathetic activity in boys; while leptin in girls was cross-sectionally associated with higher LF and LF/HF suggesting sympathetic predominance. Adiponectin was a negative cross-sectional and longitudinal predictor of parasympathetic activity in boys; but when adjusting for mean heart rate, this effect disappeared and adiponectin was a positive cross-sectional and longitudinal predictor of parasympathetic activity in girls. These results stress the importance of considering sex differences and adjustment for heart rate in testing HRV predictors. Leptin seemed disadvantageous for the autonomic balance, while adiponectin seemed advantageous for the autonomic balance in girls only. More research is needed to see whether leptin and adiponectin are interesting in cardiovascular screening/prevention or in determining the cardiovascular gain during weight loss follow-up.

Keywords: leptin, adiponectin, heart rate variability, children, longitudinal, autonomic nervous system

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INTRODUCTION

Cardiovascular diseases cause invalidity, diminish life quality and are a main cause of death in the Western world (World Health Organization, 2012). Several studies confirm that cardiovascular problems are initiated in childhood. Consequently, prevention of cardiovascular disease should start at an early age. Herein, a good understanding of the pathogenesis and risk factors is necessary, and the early detection of problems is important. In this context, cardiovascular problems can be non-invasively reflected by the autonomic balance. A dysfunctional autonomic balance, i.e., an increase in sympathetic activity and/or a decrease in parasympathetic activity, is related to cardiovascular diseases and cardiovascular mortality (Baum et al., 2013).

Since cardiovascular disease is influenced by overweight and obesity, it seems straightforward to define biomarkers related with overweight and cardiovascular health in the search of pathogenic

markers (Giannini et al., 2009; Baum et al., 2013; Mahmood et al., 2014). Overweight and obesity are associated with hyperplasia and hypertrophy of adipose tissue, which functions as an endocrine organ. After all, adipose tissue is responsible for the secretion of more than 50 hormones and signal molecules which are involved in energy homeostasis and inflammation and which are collectively referred to as adipokines. In the perspective of obesity and cardiovascular disease, the predictive power of the 16 kDa adipokine leptin and the 30 kDa cardioprotective adiponectin seems most relevant to study (Bruyndonckx et al., 2013).

Leptin is involved in energy homeostasis since it suppresses the appetite, stimulates thermogenesis, increases fatty acid oxidation, reduces plasma glucose, and reduces body fat. People with overweight and obesity have higher leptin concentrations but often become leptin resistant. This is a phenomenon whereby the increased leptin concentrations can no longer suppress appetite. The high leptin concentrations in overweight and obese people are related with the pathological changes of the vasculature in these patients: leptin is atherogenic (Harwood, 2012; Bruyndonckx et al., 2013; Charles et al., 2015).

Adiponectin is cardioprotective as it has anti-inflammatory and anti-atherogenic characteristics. It decreases the production of inflammatory cytokines, vascular cell adhesion molecules, and foam cells and induces a higher production of nitric oxide. In addition, adiponectin increases insulin sensitivity. Adiponectin is increasingly used as a biomarker of insulin sensitivity or as predictor of cardiovascular risk, since life style changes can easily influence the adiponectin concentrations in a positive way (Harwood, 2012; Caselli et al., 2013).

In this study, the association between the autonomic balance, measured by heart rate variability (HRV), and the adipokines leptin, and adiponectin is examined in healthy children. Until now, those associations have been mainly explored in adults, and most often in clinical populations. In several clinical adult populations, a negative association between adiponectin and sympathetic activity was seen, as well as a positive association with parasympathetic activity (Fasshauer et al., 2003; Takahashi et al., 2007; Boer-Martins et al., 2011; Barbosa-Ferreira et al., 2015). However, literature does not report unambiguously on the association between the autonomic balance and leptin. A positive association between leptin and sympathetic activity is suggested by some authors (Paolisso et al., 2000; Flanagan et al., 2007; Pieterse et al., 2014). Other studies revealed a negative association with sympathetic activity (Charles et al., 2015) and parasympathetic activity (Paolisso et al., 2000; Flanagan et al., 2007). In this study, we hypothesize that high leptin concentrations might be related to increased sympathetic activity, whereas adiponectin might influence the autonomic balance in favor of parasympathetic activity.

MATERIALS AND METHODS

Design

Participants of this study were Belgian children recruited for the longitudinal ChiBS study (year 2010 and 2012). Children were between 4.4 and 11.0 years old at baseline and between 6.7 and 12.2 years old at follow-up. More details on the ChiBS study and its measurements have been described elsewhere (Michels et al., 2012). Since blood withdrawal was optional, not all participant could be included in the current analysis. In 2010, 270 from the 520 children had information on all variables for the current analyses: serum values and HRV but this number was diminished to 249 after checking all confounders. Of them, 230 also had information on HRV and serum values in 2012 but this number was diminished to 223 after checking all confounders. On the examination day in 2010 and 2012, fasting blood withdrawal was executed (i.a. for leptin and adiponectin level determination) and HRV was measured. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and the project protocol was approved by the Ethics Committee of the Ghent University Hospital. A written informed consent was obtained from the parents and a verbal assent from the children.

Biomarkers: Leptin and Adiponectin

Leptin and adiponectin were measured using a venous blood sample after an overnight fast. The venipuncture was done at the level of the antecubital vein in the left arm. Serum tubes were stored at room temperature for 30 min to allow clotting. Processing of the blood samples was performed within 4 h after collection. All blood samples were centrifuged at 2,500 g for 10 min and were stored at −80°C until further analyses of the extracted serum. Leptin serum concentrations were measured in 2010 using a Meso Scale Discovery sandwich electrochemiluminescence immunoassay (inter-assay CV 2.4% for low, 3.9% for high controls; intra-assay CV 2.7% for low, 5.1% for high controls) and in 2012 using a Millipore radioimmunoassay in a certified laboratory (inter-assay CV 3.0% for low, 6.2% for high controls; intra-assay CV 3.4% for low, 8.3% for high controls). Millipore radioimmunoassay was also used to measure adiponectin serum concentrations, in both 2010 and 2012.

HRV

To define HRV, each child was individually examined in a quiet room in supine position (i.e., lying down with the face up) during 10 min. Children were asked to refrain from strenuous physical activity on the measurement day. The child was encouraged to be calm, to breath normally and not to speak or move during the 10 min of HRV measurement. The heart rate belt was fixed around the chest and measurements were started after a couple of minutes when the signal was stabilized. RR-intervals (RRI) were recorded at a sampling rate of 1,000 Hz with the elastic electrode belt Polar Wear link 31 using a Wind link infrared computer transmitter. This low-cost device has a proven validity in supine position compared to the gold standard of an electrocardiogram device ($r > 0.97$; Gamelin et al., 2006), also in children ($r > 0.99$; Gamelin et al., 2008).

The middle 5 min were manually checked on their quality and if necessary, another appropriate 5 min interval was chosen. Quality was defined as no large RRI outliers, an equidistance between consecutive RRI points and unimodal and Gaussians RRI and heart rate distribution graphics. As

such, disturbing phenomena like the Valsalva maneuvre were excluded. Data processing to obtain time-domain and frequency-domain parameters was performed with the free, professional HRV Analysis Software of the University of Kuopio, Finland (Niskanen et al., 2004). The RR series were de-trended using the Smoothness priors method with alpha = 300 and a cubic interpolation at the default rate of 4 Hz was done (Task Force, 1996). Low frequency (LF) and high frequency (HF) bands were analyzed between 0.04–0.15 and 0.15–0.4 Hz, as default (Task Force, 1996) using the autoregression method. In addition, the LF/HF ratio was calculated as approximate parameter of the sympathovagal balance. HF was expressed in power units and was used as a marker of the parasympathetic activity. LF was expressed in normalized units [=LF/(total power − very low power)] to roughly represent the sympathetic activity (although it might be a mixture of sympathetic and parasympathetic activity). In the time domain analysis, pNN50 (percentage of successive normal sinus RR intervals >50 ms) was used as marker of the parasympathetic activity. In Supplementary Tables 1–3 the results are shown for 4 other HRV parameters that do not give additional information next to the 4 others mentioned above: root mean square of successive differences (RMSSD), standard deviation of normal RR intervals (SDNN), HF in normalized units, LF in power.

Confounding Factors

Sex, age, parental education level, body fat%, negative emotions and mean heart rate were considered as potential confounding factors, based on previous research (Porges et al., 1994; Blum et al., 1997). When analyzing leptin, pubertal status was considered as extra confounding factor in 2012. The children's sex and birth date were reported by the parent. To represent socio-economic status, parental education level was assessed by questionnaire according to the International Standard Classification of Education (Unesco, 2010). Six groups (1 = low income, 6 = high income) were distinguished. Due to the limited percentage of children in the lowest groups, group 1 until 4 and groups 5 and 6 were respectively recoded in "low income" and "high income." Body fat% was reliably measured using air-displacement plethysmography (BOD POD®) in both 2010 and 2012. Children were measured following standardized procedures: twice in tight-fitting bathing suit with swim cap to rule out air trapped in clothes or hair and child-specific conversion factors were applied (McCrory et al., 1995). For negative emotions, children were questioned about their recent feelings by rating the feelings anger, anxiety, and sadness on a 0 to 10 Likert-scale (0 "not at all" to 10 "very strong;" Zimmer-Gembeck et al., 2009). To help the children understand these distinct feelings, pictures of a social skills training game for very young children were displayed next to the question. The sum of the three negative emotions was used as confounder. Pubertal status was assessed in 2012 using Tanner classification based on staging of pubic hair distribution and genital development for boys, and pubic hair distribution and breast development for girls. Due to the limited percentage of children in the Tanner stages higher than stage I, these data were recoded into two groups ("no sign of puberty" vs. "signs of puberty"). Heart rate was defined in analogy to HRV: in supine position with an electrode band and infrared computer transmission.

Statistical Analyses

Statistical analyses were performed using SPSS 23 (SPSS Inc., Chicago, IL) and all $p < 0.05$ were considered significant. The association between the biomarkers (predictor) and HRV (outcome) was tested with cross-sectional linear regression using 2010 and 2012 variables. When the regression residuals were not normally distributed, outcome variables were transformed by calculating the natural logarithm. Since all variables were available in both 2010 and 2012, the association between adiponectin and leptin (predictors) and HRV (outcome) was also tested with longitudinal mixed model linear regression. This multilevel method enables testing the longitudinal change in the slope (two measurements within the individual) by including predictor and outcome factors of the two time points; thus having the advantage of not using difference scores but slopes for longitudinal analyses. The longitudinal effect is reflected by the time*predictor variable (while also including the separate time and predictor variable in the model). Adiponectin was also tested as outcome of HRV, since literature reports unclearly about the role of adiponectin in relationship to autonomic activity.

Adjustment for confounding factors was performed in several consecutive steps. First, we adjusted for age, sex, and parental education as general confounders. Secondly, body fat% was added as additional confounder. Thirdly, negative emotions were added as additional confounder since HRV might also be influenced by stress/emotions (Porges et al., 1994; Michels et al., 2013). In case of leptin, adjustment for pubertal status was included in the analyses of 2012. At last, heart rate was included as additional confounding factor. A stepwise regression model did not show different results compared to this consecutive confounder model. Since sex interaction terms (i.e., sex*predictor) were often significant in our sample, analyses were split by sex.

RESULTS

Descriptive Characteristics

Descriptive values of leptin levels, adiponectin levels, HRV indices, and confounding factors can be found in **Tables 1A,B**. Some sex differences were detected with girls having significantly higher leptin levels at baseline and at follow-up; while pNN50 and HF at follow-up were significantly higher in boys compared to girls. When comparing data at baseline and at follow up, leptin was significantly increased over time in both sexes, while adiponectin and LF/HF ratio were significantly increased over time in girls only, and LF in boys only. Leptin was positively correlated with age, while LF and LF/HF were negatively correlated with age. At baseline, 6.74% of the boys and 10.13% of the girls were overweight or obese. At follow-up, respectively 6.54 and 12.07% of the children weighed too much. As expected, leptin was positively correlated with body fat% ($r = 0.448$ $p < 0.001$ at baseline; $r = 0.776$ $p < 0.001$ at follow-up) but adiponectin was not.

TABLE 1A | Descriptive statistics of the population.

2010				2012				≠ 2010 and 2012	
Adipokines	**♂ —*N* = 125**	**♀—*N* = 124**	**≠ ♂ & ♀**	**Adipokines**	**♂ —*N* = 107**	**♀—*N* = 116**	**≠ ♂ & ♀**	**♂**	**♀**
	Median [IQR]	**Median [IQR]**	***P***		**Median [IQR]**	**Median [IQR]**	***p***	***p***	***p***
Leptin (ng/ml)	1.02 [1.05]	1.70 [3.01]	**<0.001**	Leptine (ng/ml)	2.48 [1.69]	4.29 [3.54]	**<0.001**	**<0.001**	<0.001
Adiponectin (μg/ml)	9.19 [63]	8.56 [13]	0.325	Adiponectine (μg/ml)	14 [5.10]	15 [4.95]	0.073	0.209	**0.006**
HRV Parameters	♂	♀	**≠ ♂ & ♀**	**HRV Parameters**	♂	♀	**≠ ♂ & ♀**	♂	♀
pNN50 (%)	41 [25]	41 [29]	0.645	pNN50 (%)	44 [24]	40 [29]	**0.024**	0.526	0.623
LF (normalized units)	47 [21]	47 [17]	0.500	LF (normalized units)	47 [18]	46 [19]	0.986	**0.016**	0.484
HF (ms^2)	411 [508]	422 [505]	0.677	HF (ms^2)	462 [567]	393 [632]	**0.049**	0.152	0.201
LF/HF	1.05 [1.07]	0.98 [0.80]	0.162	LF/HF	1.25 [1.15]	1.19 [1.36]	0.985	0.616	**0.013**

♂, man; ♀, woman; N, number; [IQR], interquartile range; p, significance; HRV, Heart Rate Variability; pNN50, percentage of successive normal sinus RR intervals >50 ms; LF, low frequency; HF, high frequency; LF/HF, low frequency/high frequency. Bold indicates p < 0.05.

TABLE 1B | Descriptive statistics of the confounding factors.

2010			2012		
Confounding factors	**♂ *N* = 125**	**♀ *N* = 124**	**Confounding factors**	**♂ *N* = 107**	**♀ *N* = 116**
	% Median [IQR]			**% Median [IQR]**	**% Median [IQR]**
Age	8.00 [2.00]	8.40 [1.60]	Age	9.79 [2.30]	10.06 [2.20]
Body fat (%)	18 [6.17]	21 [9.85]	Body fat%	16 [7.60]	23 [8.78]
Mean heart rate (beats/min)	79 [13]	82 [9.71]	Mean heart rate (beats/min)	75 [15]	79 [14]
Negative emotions (0–30)	6.00 [8.00]	7.50 [7.00]	Negative emotions (0–30)	4.00 [6.00]	5.00 [7.00]
SES (low/high)	23.20% low 76.80% high	22.58% low 77.42% high	Tanner stadium (no signs/puberty signs)	76.64% no signs 23.36% puberty signs	66.38% no signs 33.62% puberty signs

♂, man; ♀, woman; N, number; [IQR], interquartile range; SES, socioeconomic status.

Associations of Leptin with the Autonomic Balance

Results of leptin as predictor of the autonomic balance at baseline are shown in **Table 2**. In girls, a positive significant association was found for leptin with LF and LF/HF, also after adjustment for confounding factors. At follow-up (see **Table 3**), leptin was negatively associated with pNN50 and HF in boys. After adjustment for body fat%, the significance for HF disappeared, but the association with pNN50 remained all the way. Longitudinally, a negative significant effect of leptin on pNN50 and HF was detected in boys (**Table 4**).

Association of Adiponectin with the Autonomic Balance

As seen in **Table 2**, there were no significant associations between adiponectin and HRV at baseline. At follow up (see **Table 3**), a negative association between adiponectin and HF was seen in boys, which disappeared after adjusting for mean heart rate. However, significant associations in girls were seen after adjustment for mean heart rate: positive association with HF and borderline significant with pNN50. Longitudinal analyses (**Table 4**) showed similar results as the 2012 cross-sectional analyses. The negative effect on pNN50 and HF in boys disappeared after adjustment for mean heart rate. In girls, a positive effect of adiponectin on HF was visible after adjustment for mean heart rate. The analyses considering adiponectin as outcome of HRV (data not shown), did not show any significant results.

DISCUSSION

In a group of Belgian primary school children, we aimed to examine the cross-sectional and 2-year longitudinal associations between the adipokines leptin and adiponectin and the autonomic balance as measured by HRV. The associations differed between boys and girls and the tested confounders seemed to have an important role in the associations. This study confirmed that leptin is positively associated with sympathetic activity, but only in girls and cross-sectionally. Besides, high leptin concentration was a cross-sectional and longitudinal predictor of decreased parasympathetic activity, mainly in boys. The hypothesis of adiponectin as a positive predictor of parasympathetic activity was also confirmed in this cross-sectional and longitudinal study, but only in girls and after adjustment for heart rate. Contrary,

Table 2 | Linear regression with adipokines predicting heart rate variability, 2010 ($N = 249$).

	♀/♂	pNN50			LFNORMALIZED			HFPOWER			LF/HF		
		ADJR²	β	*P*	ADJR²	β	*P*	ADJR²	β	*P*	ADJR²	β	*P*
Leptin	♂	−0.005	0.055	0.540	−0.008	0.003	0.971	−0.008	−0.015	0.868	−0.004	−0.064	0.478
	♀	−0.008	0.018	0.840	0.013	0.144	0.110	−0.007	−0.028	0.759	0.014	0.149	0.100
Model 1	♂	0.010	0.026	0.777	−0.014	0.020	0.824	−0.005	−0.032	0.727	−0.002	−0.045	0.625
	♀	−0.012	0.007	0.942	0.014	0.171	0.064	−0.014	−0.036	0.694	0.021	0.178	0.052
Model 2	♂	0.004	0.032	0.724	−0.020	0.014	0.879	0.012	−0.012	0.893	−0.004	−0.054	0.557
	♀	−0.013	0.100	0.455	0.033	0.342	**0.010**	−0.021	0.007	0.958	0.052	0.384	**0.004**
Model 3	♂	−0.001	0.033	0.723	−0.022	0.014	0.883	0.005	−0.012	0.895	−0.011	−0.054	0.557
	♀	0.074	0.160	0.215	0.067	0.302	**0.021**	0.125	0.084	0.505	0.105	0.336	**0.009**
Model 4	♂	0.498	−0.065	0.327	0.003	0.039	0.676	0.335	−0.091	0.228	0.050	−0.018	0.838
	♀	0.556	−0.052	0.567	0.219	0.424	**0.001**	0.484	−0.100	0.310	0.245	0.453	**<0.001**
Adiponectin	♂	−0.007	−0.026	0.769	−0.006	−0.045	0.615	−0.008	0.018	0.840	0.001	−0.089	0.325
	♀	−0.003	0.074	0.417	−0.008	0.024	0.793	−0.006	0.047	0.608	−0.008	−0.026	0.775
Model 1	♂	0.011	−0.045	0.619	−0.013	−0.035	0.699	−0.006	0.015	0.868	0.003	−0.082	0.363
	♀	−0.007	0.077	0.399	−0.014	0.030	0.746	−0.013	0.051	0.577	−0.010	−0.016	0.857
Model 2	♂	0.005	−0.042	0.643	−0.018	−0.039	0.673	0.013	0.025	0.782	0.001	−0.087	0.339
	♀	−0.012	0.077	0.400	−0.023	0.030	0.748	−0.018	0.051	0.578	−0.018	−0.016	0.858
Model 3	♂	0.001	−0.035	0.706	−0.020	−0.051	0.579	0.006	0.031	0.738	−0.005	−0.096	0.299
	♀	0.070	0.089	0.310	0.024	0.020	0.822	0.127	0.067	0.433	0.052	−0.028	0.755
Model 4	♂	0.494	0.030	0.646	0.006	−0.068	0.457	0.334	0.084	0.268	0.064	−0.121	0.176
	♀	0.562	0.083	0.168	0.137	0.023	0.784	0.483	0.062	0.346	0.152	−0.025	0.766

♂, man; ♀, woman; N, number; pNN50, percentage of successive normal sinus RR intervals >50 ms; LF, low frequency; HF, high frequency; LF/HF, low frequency/high frequency; ADJR², adjusted r²; β, standardized regression coefficient; p, significance—Model 1, correction for age, socioeconomic status; Model 2, Model 1 + body fat%; Model 3, Model 2 + negative emotions; Model 4, Model 3 + mean heart rate. Bold indicates $p < 0.05$.

adiponectin was a negative predictor of parasympathetic activity in boys but no longer after adjustment for heart rate.

Association of Leptin and the Autonomic Balance

In girls, there was a positive association of leptin with LF/HF and LF at baseline, roughly representing higher sympathetic activity. Interestingly, the association was stronger after correction for the tested confounding factors (e.g., from $\beta = 0.384$ to $\beta = 0.453$ for LF/HF), with the biggest impact after adjusting for body fat% and mean heart rate. The confounding role of body fat% seems straightforward as leptin is produced by adipose tissue increasingly with bigger or more fat cells (Fried et al., 2000) and as weight decrease has previously been related to HRV improvements probably due to changed free fatty acid levels (Li et al., 2009). Those findings also imply the importance of so-called negative confounders: confounders that cause an underestimation of the association. For example, age was negatively correlated with LF and LF/HF in this study and significantly positively with leptin.

The positive association between leptin and LF or LF/HF in girls is analogous to the findings of Flanagan et al. who showed a positive association in adult women between leptin and sympathetic activity (Flanagan et al., 2007), although others found a positive association between leptin and sympathetic activity in men (Paolisso et al., 2000; Pieterse et al., 2014). In contrast, a negative association between leptin and sympathetic activity was found in both women and men with a body fat% >25.5%. This is remarkable since body fat% is expected to have a positive correlation with both leptin and sympathetic activity (Charles et al., 2015).

Still, a positive leptin-sympathetic association seems logic from a physiological viewpoint. Leptin stimulates energy-expenditure in fat mass and enhances sympathetic activity in non-thermogenic organs and glands (e.g., kidney and adrenal gland) by an increase in noradrenalin concentrations, the neurotransmitter of the sympathetic nervous system (Vinik et al., 2011). The fact that the effects of leptin are mediated by the sympathetic nervous system, is also supported by a study in animals, where blockage of adrenal activity destroyed the effects of leptin (Vinik et al., 2011).

At follow-up and longitudinally, we found a significant and negative association between leptin and parameters of parasympathetic activity (HF and pNN50) in boys. The association with pNN50 was independent of body fat% and mean heart rate. This association was also found by Flanagan et al. in women and was also reported by Paolisso et al. in men (Paolisso et al., 2000; Flanagan et al., 2007). Our study did not show this significant association in girls. The significantly higher values for pNN50 and HF in boys at follow-up could possibly explain this discrepancy. The inverse association between leptin

Table 3 | Linear regression with adipokines predicting heart rate variability, 2012 ($N = 223$).

	♀/♂	pNN50			LFnormalized			HFpower			LF/HF		
		ADJR²	β	*P*	ADJR²	β	*P*	ADJR²	β	*P*	ADJR²	β	*P*
Leptin	♂	0.145	−0.392	**<0.001**	−0.006	0.062	0.526	0.067	−0.276	**0.004**	−0.009	0.005	0.958
	♀	−0.005	−0.057	0.542	−0.009	−0.009	0.920	−0.008	−0.029	0.754	−0.008	−0.018	0.849
Model 1	♂	0.144	−0.423	**<0.001**	0.020	0.115	0.255	0.087	−0.325	**0.001**	0.046	0.074	0.459
	♀	−0.019	−0.074	0.451	−0.012	0.019	0.844	−0.026	−0.035	0.725	−0.017	0.005	0.961
Model 2	♂	0.135	−0.435	**0.002**	0.015	0.043	0.772	0.086	−0.232	0.103	0.043	−0.014	0.925
	♀	0.001	0.139	0.368	−0.019	−0.034	0.827	0.017	0.248	0.107	−0.024	−0.057	0.713
Model 3	♂	0.142	−0.408	**0.004**	0.006	0.050	0.738	0.077	−0.228	0.116	0.036	−0.004	0.979
	♀	−0.006	0.137	0.377	0.001	−0.027	0.863	−0.008	0.247	0.110	−0.014	−0.051	0.741
Model 4	♂	0.133	−0.407	**0.004**	0.021	0.042	0.775	0.069	−0.226	0.120	0.063	−0.013	0.929
	♀	0.012	0.125	0.415	−0.008	−0.029	0.850	0.015	0.239	0.122	−0.019	−0.056	0.719
Model 5′	♂	0.557	−0.210	**0.041**	0.056	−0.024	0.869	0.452	−0.039	0.730	0.086	−0.078	0.592
	♀	0.637	0.061	0.514	0.145	0.010	0.942	0.474	0.182	0.106	0.129	−0.015	0.920
Adiponectin	♂	0.024	−0.182	0.060	−0.002	0.086	0.379	0.071	−0.283	**0.003**	0.001	0.098	0.314
	♀	−0.009	0.013	0.894	−0.007	−0.042	0.658	−0.004	0.070	0.457	−0.007	−0.039	0.678
Model 1	♂	0.006	−0.185	0.064	0.017	0.098	0.319	0.073	−0.292	**0.003**	0.054	0.113	0.242
	♀	−0.024	0.018	0.851	−0.009	−0.053	0.577	−0.022	0.072	0.457	−0.015	−0.048	0.614
Model 2	♂	0.073	−0.155	0.105	0.022	0.086	0.384	0.133	−0.266	**0.005**	0.054	0.103	0.289
	♀	−0.006	0.009	0.925	−0.017	−0.051	0.595	−0.003	0.062	0.514	−0.023	−0.047	0.629
Model 3	♂	0.101	−0.187	0.053	0.012	0.084	0.405	0.132	−0.279	**0.004**	0.045	0.098	0.320
	♀	−0.013	0.015	0.880	0.004	−0.069	0.470	−0.011	0.066	0.495	−0.012	−0.062	0.524
Model 5	♂	0.538	−0.014	0.840	0.056	0.024	0.816	0.467	−0.128	0.096	0.085	0.041	0.682
	♀	0.647	0.106	0.066	0.158	−0.115	0.197	0.483	0.145	**0.038**	0.140	−0.107	0.235

♂, man; ♀, woman; N, number; pNN50, percentage of successive normal sinus RR intervals >50 ms; LF, low frequency; HF, high frequency; LF/HF, low frequency/high frequency; ADJR², adjusted r^2; β, standardized regression coefficient; p, significance; Model 1, correction for age, socioeconomic status; Model 2, Model 1 + body fat%; Model 3, Model 2 + negative emotions; Model 4, Model 3 + pubertal status (only relevant for leptin); Model 5′ (for leptin), Model 4 + mean heart rate; Model 5, Model 3 + mean heart rate. Bold indicates $p < 0.05$.

Table 4 | Mixed model regression, longitudinally.

	♀/♂	pNN50		LFnormalized		HFpower		LF/HF	
		b	*P*	*b*	*P*	*b*	*P*	*b*	*P*
LEPTIN									
Model 1	♂	−2.857	**<0.001**	0.378	0.525	−0.035	**0.028**	0.002	0.893
	♀	−0.259	0.563	−0.359	0.344	0.002	0.884	0.008	0.346
Model 2	♂	−2.702	**0.001**	0.139	0.839	−0.015	0.420	−0.006	0.687
	♀	−0.142	0.753	−0.272	0.481	0.001	0.896	−0.006	0.490
Model 3	♂	−1.607	**0.016**	−0.257	0.732	0.012	0.473	−0.016	0.292
	♀	0.140	0.614	0.055	0.893	0.002	0.799	0.002	0.798
ADIPONECTIN									
Model 1	♂	−0.798	**0.023**	0.306	0.297	−0.023	**0.002**	0.006	0.304
	♀	0.114	0.786	−0.122	0.718	0.011	0.256	−0.003	0.681
Model 2	♂	−0.711	**0.039**	0.279	0.343	−0.021	**0.005**	0.006	0.347
	♀	0.085	0.838	−0.120	0.722	0.011	0.277	−0.003	0.685
Model 3	♂	0.023	0.928	0.123	0.674	−0.007	0.232	0.001	0.803
	♀	0.323	0.154	−0.249	0.431	0.017	**0.015**	−0.006	0.424

*♂, man; ♀, woman; N, number; pNN50, percentage of successive normal sinus RR intervals >50 ms; LF, low frequency; HF, high frequency; LF/HF, low frequency/high frequency; b, regression coefficient of the time*predictor parameter to show the longitudinal relation—p, significance; Model 1, correction for age, socioeconomic status; Model 2, Model 1 + body fat%; Model 3, Model 2 + mean heart rate. Bold indicates $p < 0.05$.*

and the parasympathetic nervous system, also known as the anti-inflammatory component of the autonomic system, is supported by the bi-directional relationship between leptin and inflammation. After all, leptin increases the competence of the immune system and the synthesis of leptin is stimulated by inflammatory cytokines (Jung et al., 2012).

Association of Adiponectin and the Autonomic Balance

The cross-sectional and longitudinal results confirmed the hypothesis that adiponectin is a positive predictor of parasympathetic activity, but this was only true in girls and after adjustment for heart rate. Contrary, adiponectin was a negative predictor of parasympathetic activity in boys but no longer after adjustment for heart rate.

Studies exploring the association between adiponectin and the autonomic balance in healthy subjects are limited. However, it is expected that the physiological mechanisms in healthy subjects are similar as in patients with metabolic and cardiovascular diseases, though extrapolation of study results of patients into a healthy population should be done with caution. Literature mainly reports a positive association between adiponectin and parasympathetic activity (Fasshauer et al., 2003; Takahashi et al., 2007; Boer-Martins et al., 2011; Barbosa-Ferreira et al., 2015). This positive link between adiponectin and parasympathetic activity was also seen in girls in our cross-sectional and longitudinal analyses but only after correcting for mean heart rate. The results after correction for mean heart rate correspond to what is seen in literature and also seem logic considering the knowledge about adiponectin (lower adiponectin levels as well as lower parasympathetic activity in people with overweight and obesity). Consequently, correction for heart rate seems to have an important influence on the association between HRV and adiponectin and supports the hypothesis that HRV cannot be interpreted correctly without taking heart rate into account (Sacha and Pluta, 2005, 2008; Billman, 2013a).

Based on literature, it is unclear whether adiponectin should be considered as predictor or outcome of HRV. Several studies suggest that sympathetic hyperactivity diminishes the expression of adiponectin in fat tissue (Delporte et al., 2002; Fasshauer et al., 2003; Wakabayashi and Aso, 2004; Takahashi et al., 2007; Hoyda et al., 2009). However, there is doubt whether the low adiponectin concentrations are the cause or consequence of the sympathetic hyperactivity (Takahashi et al., 2007; Hoyda et al., 2009). On the other hand, some studies support the hypothesis that adiponectin is a predictor of HRV. For example, Hoyda et al. described the autonomic effects of adiponectin by depolarization of parvocellular neurons in the paraventricular nucleus and emphasized the importance of adiponectin in the energy and autonomic homeostasis regulation (Hoyda et al., 2009). Our longitudinal analyses confirm adiponectin as predictor of HRV rather than HRV as predictor of adiponectin. Consequently, HRV cannot be used as non-invasive measure for adiponectin levels.

Remarkable in the results of this study is the lower amount of significant findings at baseline compared to follow-up. At first sight, it might be because the follow-up study comprised somewhat less participants. Though, even less associations were significant when analyzing the group of children with both data at baseline and follow up. A possible explanation for this discrepancy could be the difference in age: children were ∼2 years older at follow-up. Biological changes in puberty could influence the physiology of the association between adiponectin and the autonomic balance. Possibly, the fat tissue is endocrinologically more active in puberty. This hypothesis is supported by our findings: adiponectin concentrations were significantly higher at follow-up in comparison to baseline, mainly in girls. When comparing boys and girls, significant associations differed as well. As we already suggested for the association between HRV and leptin, this could be due to the significant higher values for pNN50 and HF in boys at follow-up.

Strengths and Limitations

Compared to existing research, our study had several strengths. First of all, the size of the studied cohort (at baseline 249; at follow-up 223 children) is larger than most other published studies. Secondly, this study responds to the lack of studies examining the association between the autonomic balance and the adipokines leptin and adiponectin in healthy people. Especially a study examining this association in children is unique. Another strength is the correction for confounding factors. This study distinguishes itself by correcting for mean heart rate. Splitting the analyses by sex is also an added value of this study. A final strength is the longitudinal analysis to suggest some directionality.

Of course, this study is not without limitations. First of all, results might be difficult to generalize because of a selection bias with more high-educated parents. At baseline, the selected population consisted of less girls and older children than in the total cohort of the ChiBS-study. Because of the exclusion of low-quality data and some technical problems with the measurements, HRV data were not available for all children. Furthermore, LF is no perfect sympathetic parameter as it reflects both sympathetic and parasympathetic activity; and also the traditional interpretation of LF/HF has been criticized (Billman, 2013b). Also, monitoring respiration might be recommended for the frequency domain measures. In the longitudinal analyses, there is the limitation that the leptin analysis kit was different at baseline and at follow-up but a previous study showed that different leptin analysis kits yielded almost indistinguishable concentrations (Carlson et al., 1999).

CONCLUSION

In conclusion, high leptin and low adiponectin are unfavorable for the autonomic balance as measured with HRV and consequently for the cardiovascular risk, even during childhood. These associations were independent of body fat%. More research is needed to see whether determination of leptin and adiponectin in blood can be used as a measure of cardiovascular health in overweight and obese children. For example, determination of these biomarkers could be useful in determining the cardiovascular gain and thus in increasing motivation during

the follow-up of weight loss. In addition, we suggest to examine whether treatments focusing on high leptin and low adiponectin levels are favorable for someone's cardiovascular health. Those studies could increase the understanding of cardiovascular disease pathogenesis.

AUTHOR CONTRIBUTIONS

RV has performed the statistical analyses and made a manuscript draft. NM designed the hypothesis, helped in data collection, guided in statistical analyses, and edited the draft.

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SUPPLEMENTARY MATERIAL

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Non-linear Heart Rate Variability as a Discriminator of Internalizing Psychopathology and Negative Affect in Children With Internalizing Problems and Healthy Controls

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Background: Internalizing psychopathology and dysregulated negative affect are characterized by dysregulation in the autonomic nervous system and reduced heart rate variability (HRV) due to increases in sympathetic activity alongside reduced vagal tone. The neurovisceral system is however, a complex nonlinear system, and nonlinear indices related to psychopathology are so far less studied in children. Essential nonlinear properties of a system can be found in two main domains: the informational domain and the invariant domain. sample entropy (SampEn) is a much-used method from the informational domain, while detrended fluctuation analysis (DFA) represents a widely-used method from the invariant domain. To see if nonlinear HRV can provide information beyond linear indices of autonomic activation, this study investigated SampEn and DFA as discriminators of internalizing psychopathology and negative affect alongside measures of vagally-mediated HRV and sympathetic activation.

Material and Methods: Thirty-Two children with internalizing difficulties and 25 healthy controls (aged 9–13) were assessed with the Child Behavior Checklist and the Early Adolescent Temperament Questionnaire, Revised, giving an estimate of internalizing psychopathology, negative affect and effortful control, a protective factor against psychopathology. Five minute electrocardiogram and impedance cardiography recordings were collected during a resting baseline, giving estimates of SampEn, DFA short-term scaling exponent α_1, root mean square of successive differences (RMSSD), and pre-ejection period (PEP). Between-group differences and correlations were assessed with parametric and non-parametric tests, and the relationships between cardiac variables, psychopathology and negative affect were assessed using generalized linear modeling.

Results: SampEn and DFA were not significantly different between the groups. SampEn was weakly negatively related to heart rate (HR) in the controls, while DFA was moderately negatively related to RMSSD in both groups, and moderately positively related to HR in the clinical sample. SampEn was significantly associated

with internalizing psychopathology and negative affect. DFA was significantly related to internalizing psychopathology.

Conclusions: Higher invariant self-similarity was linked to less psychopathology. Higher informational entropy was related to less psychopathology and less negative affect, and may provide an index of the organizational flexibility of the neurovisceral system.

Keywords: non-linear time-series analysis, HRV, sample entropy, RMSSD, detrended fluctuation analysis, pre-ejection period, anxiety, depression

INTRODUCTION

Internalizing psychopathologies like anxiety and depression are among the costliest and most prevalent disorders in society (Pincus and Pettit, 2001; Kessler, 2012). One of the reasons for this is the early debut (Kessler and Walters, 1998; Kessler et al., 2007) and high risk of carrying a psychiatric disorder into adulthood (Pine et al., 1998; Pine, 1999; Kessler, 2012). Dysregulated negative affect, the tendency to repeatedly experience negative affect and distress (Watson et al., 1994), may be a common component in both anxiety and depression and can increase the risk of internalizing psychopathology (Watson et al., 1995). Not only are anxiety and depression risk factors for later psychopathology and suicide (Beesdo et al., 2009; Kessler, 2012; Maughan et al., 2013), but they may also increase the likelihood of later cardiovascular disease and mortality through autonomic nervous system (ANS) dysregulation (Chalmers et al., 2014; Koenig et al., 2016; Kemp et al., 2017).

The neurovisceral integration model (Thayer and Lane, 2000, 2009) states that internalizing psychopathology and dysregulated negative affect is characterized by an imbalance between positive and negative feedback loops in the neurocardiac system and the central autonomic network, an internal regulatory network consisting of structures like the amygdala, hypothalamus, nucleus of the tractus solitarius and the insular cortex (Benarroch, 1993). In this model stressful internal or external stimuli lead to hypoactivation of the frontal cortex, resulting in vagal withdrawal. As the vagal nerve ordinarily works as a brake on sympathetic activation, this withdrawal readies the organism for threat through increased sympathetic nervous system (SNS) activation. In the short term, and in the face of actual threat, this reaction leads to adaptation to environmental demands and increased chances of survival. In the case of anxiety or emotional dysregulation, a chronic disinhibition of the SNS in the face of non-threatening stimuli instead leads to difficulties with adaptive and flexible behavior as well as health problems (Thayer and Friedman, 2004).

Dysregulation of affect, anxiety and depression is associated with dysregulation in the ANS as indexed by a loss of heart rate variability (HRV), in both adult (Kemp et al., 2010; Chalmers et al., 2014) and child (Koenig et al., 2016; Paniccia et al., 2017) populations. Therefore, HRV has been proposed as a general biomarker of psychopathology and dysregulated negative affect (Beauchaine and Thayer, 2015). So far, most research on HRV is based on methodology calculating variability in the time- or frequency domain, giving a measure of average or magnitude values for variability using linear approaches to signal analysis (Laborde et al., 2017). The past decades have seen an increase in more mathematically-sophisticated nonlinear methods for cardiac analysis (Sassi et al., 2015). This is important for several reasons.

First, complex adaptive systems exhibit features of high organization combined with the capability for high input-driven variability to allow for a balance between predictability of behavior and flexible adaptability (Tyukin, 2011). There is general agreement that the aim of the neurocardiac system is to support flexible and adaptive interaction with the environment while balancing internal and environmental demands, not least in the social domain (Porges, 1995; Thayer and Lane, 2000; Beauchaine, 2001). Complex adaptive systems dependent on, and interacting with, external factors typically exhibit nonlinear feedback processes (Tyukin, 2011).

Second, apart from being embedded in a complex context, the human neurocardiac system is also a complex physiological system. The heartbeat is influenced by the central autonomic network, SNS and parasympathetic nervous system (PNS) activation, baroreflexes, respiration and frontal influence, as well as highly interconnected neurons in the heart (Randall et al., 1996; Berntson et al., 2007; Wake and Brack, 2016). The subsystems affecting the heartbeat are reciprocally interacting through positive (excitatory) and negative (inhibitory) feedback loops, and afferent and efferent connections. The presence of multiple reciprocally-interacting feedback loops across different levels (e.g., heart, autonomic and central nervous systems, internal, external) gives rise to nonlinear processes (Glass and Mackey, 1988; Bertuglia and Vaio, 2005; Voss et al., 2009). Nonlinear systems and processes cannot be captured properly using only linear methodology (Glass and Mackey, 1988; Bertuglia and Vaio, 2005; West, 2014) and a nonlinear approach should be taken for cardiac analysis (Young and Benton, 2015).

Essential nonlinear properties of any system can be found in five main domains. These are the following: invariant (analysis of fractal or scale-free self-similarity), informational (entropy-methods), statistical (for instance, symbolic dynamics), geometrical (methods like Poincaré or recurrence plots) and energetic (for example time-irreversibility measures) (Voss et al., 2009; Bravi et al., 2011; Sassi et al., 2015). According to a 2015 consensus-report on nonlinear HRV, the invariant and informational domains represent the most promising areas to

investigate (Sassi et al., 2015). Both have also been shown to relate to internalizing psychopathology (de la Torre-Luque et al., 2016).

detrended fluctuation analysis (DFA) is one of the most widely-used methods within the invariant domain. Through DFA the scale-invariant self-similarity, or fractal properties, of a time series can be established (Goldberger et al., 2002). A system high in self-similarity shows the same patterns of organization at different scales of magnification. Fractals are ubiquitous in biological systems, perhaps the best-known examples being those of branching in trees, the arteries of the body or the pulmonary system. A time series can also be analyzed for self-similar correlations over shorter and longer intervals to establish scale-invariant properties. The healthy heartbeat time series shows scale-invariant self-similarity, and a loss of self-similarity in the cardiac time series occurs in disease and aging (Goldberger et al., 2002; Lipsitz, 2004; Perkiömäki, 2011; Sassi et al., 2015).

The informational domain includes specific algorithms to estimate the entropy in a given time series (Bravi et al., 2011; Sassi et al., 2015). Entropy refers to the rate of new information a system generates as it evolves. Highly regular, predictable systems do not generate much new information, and are therefore characterized by low values of entropy. Complex systems, on the other hand, behave in more unpredictable ways, display irregular behavior, and show high entropy values. In trauma or disease the heartbeat generally shows a decrease in entropy consistent with a loss of information-based complexity, although sometimes high entropy rates can be a marker of pathological disorder as well, as seen in for instance heart arrhythmias (Costa et al., 2005).

Non-linear HRV-measures have shown promise as markers of internalizing psychopathology, generally linking lower information-based complexity, lower scale-invariant self-similarity, or less-ordered complexity to disorder (de la Torre-Luque et al., 2016). A recent review of HRV-research in children and adolescents related to internalizing psychopathology shows that studies using nonlinear methods still represent a small section of the published research (Paniccia et al., 2017).

This study aims to investigate the possible relationship between two key nonlinear cardiac properties, scale-invariant self-similarity and entropy, as they relate to internalizing psychopathology and negative affect in children with internalizing difficulties and healthy controls. We will use one measure of scale-invariant nonlinear HRV, short-term detrended fluctuation analysis (DFA-α_1), and one metric from the information-based entropy domain, sample entropy (SampEn). To further knowledge about the clinical utility of nonlinear cardiac complexity we want to see if SampEn or DFA-α_1 can provide information not inherent in more traditional HRV measures, by modeling them alongside the root mean square of successive differences (RMSSD), a measure of vagally-mediated high-frequency HRV. As the neurovisceral integration model describes dysregulation in the neurovisceral system as a disinhibition of SNS activity we also include pre-ejection period (PEP), a measure of sympathetically modulated contractility of the heart (Newlin and Levenson, 1979). Finally, we investigate the relationship between the nonlinear HRV measures and the subscales of the Child Behavior Checklist (CBCL) (Achenbach and Rescorla, 2001) related to internalizing psychopathology and the subscales in the Early Adolescent Temperament Questionnaire, Revised (EATQ-R) (Ellis and Rothbart, 2001) related to negative affect. Here we also include the Effortful Control scale from the EATQ-R as a measure that is linked to risk for psychopathology and frontal executive function (Nigg, 2006; Verstraeten et al., 2009).

Hypotheses

H1: Nonlinear HRV should differ between the two groups.

H2: Higher nonlinear HRV should be related to less internalizing psychopathology, less negative affect and more effortful control.

H3: Nonlinear HRV, represented by SampEn and DFA-α_1, should provide explanatory power beyond linear PNS and SNS measures.

MATERIALS AND METHODS

Sample and Participant Selection

The study was part of a larger study on child psychotherapy (also described in Fiskum et al., 2017) and had a between-groups design consisting of a clinical and a control group assessed with electrocardiography (ECG) and impedance cardiography (ICG), along with parental reports of emotional and clinical problems.

Forty children between 9 and 13 years were referred to the study from parents, mental health providers, child protective services, general practitioner doctors and school nurses. The children were selected for inclusion in the clinical group based on scores on the CBCL above the normal range in the internalizing spectrum (anxiety or depression). Four children were excluded due to normal CBCL scores.

The children were screened for medicine use and two children were excluded for the use of allergy and asthma medicine. Other exclusion criteria were severe learning difficulties and severe co-morbid mental or physical disorders. One child was excluded due to a congenital heart defect, and one child was excluded due to possible psychotic symptoms. The clinical group consisted of 32 children (16 girls). Mean age was 10.9 years (SD 1.3).

Twenty-eight children between 9 and 13 years were recruited to the control group mainly through information at parental meetings at schools in Trondheim. Eight children were recruited through convenience sampling. The inclusion criteria were normal range scores on the CBCL and no known history of contact with mental health services for any cause. In addition, the same exclusion criteria as for the clinical group were applied. One child was excluded due to a referral to mental health services for a possible developmental disorder, one child due to a clinical range CBCL score, and one child due to medicine use. The full control group consisted of 25 children (14 girls). Mean age was 10.3 years (SD 1.2). The groups did not differ significantly in age as measured with a Mann-Whitney U-test ($U = 286$, $p = 0.067$).

The children were recruited from Trondheim (47 children) and Oslo (10 children) and included 53 Caucasian children and one Asian (clinical group), one Middle Eastern (control group) and two African heritage children (one in each group). Thirty-five children lived in a two-parent household, 13 lived with a

single parent, and 9 did not say. In the clinical group 24 mothers and 20 fathers had completed higher education (defined as more than 12 years of education), while this was the case for 21 mothers and 18 fathers in the control group. There were no statistical differences between the groups on gender balance ($U = 376$, $p = 0.66$), type of household ($U = 255$, $p = 0.58$), maternal ($U = 307$, $p = 0.18$) or paternal ($U = 278$, $p = 0.30$) education.

The participants and their parents were given oral and written explanations of the study and the procedure involved, and parents signed a written consent. The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics, and was in compliance with the Helsinki Declaration (World Medical Association, 2013). Anonymous data from the project will be uploaded to the Norwegian Centre for Research Data (www.nsd.uib.no) upon completion, likely at the end of 2020.

Assessments and Measures

The Child Behavior Checklist

The Child Behavior Checklist 6-18 parent rating form (Achenbach and Rescorla, 2001) is a clinical rating scale giving an age- and gender-adjusted assessment of a wide array of mental health concerns. A total of 120 items describing problematic behavior is rated by a parent on a 3-point Likert scale, where 0 means that the item is *not true*, 1 means the item is *somewhat or sometimes true*, and 2 means it is *very often true / very true*.

The Internalizing Problems scale in the CBCL provides a scaled score of internalizing psychopathology based on the total problematic behavior reported along the dimensions of anxiety, affective problems and somatization, and reflects the child's total dimensional load on common childhood internalizing spectrum mental disorders. The CBCL is a significant predictor of later disturbance in children between 4 and 11 years over an eight-year (Ferdinand and Verhulst, 1995) and 14-year span (Hofstra et al., 2002), and corresponds well with clinical judgment of psychopathology by health-care professionals (Verhulst and van der Ende, 1991). In the present study we focused on the Internalizing Problems scale (33 items), as well as two clinical scales related to internalizing disorder: Affective Problems (13 items, Cronbach's $\alpha = 0.83$) and Anxiety Problems (6 items, Cronbach's $\alpha = 0.75$).

The Early Adolescent Temperament Questionnaire, Revised

The Early Adolescent Temperament Questionnaire, Revised (Ellis and Rothbart, 2001) is a 62-item questionnaire that assesses temperamental and behavioral dimensions in adolescents aged 9-15. Items are rated on a 5-point Likert scale ranging from 1 (*almost always untrue*) to 5 (*almost always true*). The Negative Affect scale used in this study comprises a mean score calculated from scores on temperamental subscales Frustration (6 items), Shyness (5 items), Fear (6 items) and behavioral subscales Aggression (7 items) and Depressive Mood (5 items), reflecting negative emotional reactivity and lability as well as depressive and fearful mood states (Snyder et al., 2015). The scale Effortful Control consists of the subscales Attention (6 items), Inhibitory Control (5 items) and Activation Control (7 items), assessing frontally-mediated self-regulatory capacities. There was no Norwegian translation of the EATQ-R for the 9 to 15-year-old age group so two of the authors performed a translation which was then back-translated by an independent translator and finally approved by one of the original creators of the EATQ-R (L. K. Ellis, personal communication, June 2nd, 2014). The Cronbach's α for the different subscales in the translated version of the EATQ-R were comparable to the original version; Aggression: 0.80 (original scale 0.79); Depressive Mood: 0.76 (0.74); Frustration: 0.84 (0.83); Fear: 0.71 (0.69); Shyness 0.87 (0.72); Activation Control 0.88 (0.66); Attention 0.75 (0.65); and Inhibitory Control 0.65 (0.86).

Whether mothers or fathers completed the CBCL and the EATQ-R varied between families and therefore the parental ratings represent ratings from both mothers and fathers. Parental ratings have been shown to be generally consistent, but as some studies have shown that fathers are better at predicting future problems (Webster-Stratton, 1988; Hay et al., 1999) the paternal rating was chosen when both parents had responded.

Psychophysiological Protocol

Before the psychophysiological recordings began all children were in the lab for a minimum of 30 min, the first 15 of which at least one parent was also present. The children and parents received a briefing about the equipment, the protocol and the right to terminate. Following this, parents were escorted to a private waiting area and briefed about the study questionnaires. The children were instructed to use the bathroom, put on a t-shirt, and remove their shoes. The children were weighed on a freestanding scale (Coline brand). Height was measured against a wall. The electrode sites were then lightly washed and scrubbed (NuPrep scrub gel). Three Ag/AgCl electrodes were attached in a lead-II ECG configuration on the clavicles and bottom rib. Four sets of double-Ag/AgCl electrodes were attached to the sides of the upper torso and neck for the ICG. A Biopac TSD201 respiratory belt was also fastened around the torso to monitor respiration. The last 10 min before the recording began the children were seated quietly in the experimental chair, while the experimenter readied the equipment. The children were instructed to sit as quietly as possible with their eyes open. The children were not instructed to focus on their breathing in any way. The recordings were controlled by a Cedrus Stimtracker, and were 300 s long.

The Biopac MP150 system with AcqKnowledge version 4.4 software and the ECG 100C amplifier was used for the ECG recordings. The sampling rate was set to 1,000 Hz, with a 50 Hz notch filter to control for power-line artifacts. A 1.0 Hz high pass and a 35 Hz low-pass filter was also applied to the ECG signal. The ICG was recorded with the MP150 system and a wireless Biopac Bionomadix transmitter. On acquisition a 10 Hz low-pass filter was applied to the ICG signal.

The recordings took place in Oslo and Trondheim, using the same equipment and the same protocol, conducted by female experimenters. The locations were set up to be as identical as possible, and were temperature controlled, quiet, and visually sparse. To rule out any confounding effects of location, location

was included as a controlling variable in the generalized models. As the preliminary models did not show any signs that location was a significant variable, it was not included in the final models.

Biometric Analysis

Pre-processing

For analysis of the five-min recording of ECG data HRV-software Kubios version 3.0.1 (Tarvainen et al., 2014) was used, with a 500-lambda smoothness prior filter to reduce the influence of very low frequency components and drift. The data was inspected visually for artifacts and misidentified beats were corrected manually in Kubios by shifting the indicator for the R-point, using the RR tachogram and the raw ECG signal to locate the correct point in the ECG-signal. Visually identified ectopic beats were corrected with interpolated RR-values using the automatic artifact correction option in Kubios. No additional beats were corrected beyond those already identified as artifacts. A further description of the Kubios artifact-correction procedure can be found in the Kubios manual (Tarvainen et al., 2017). To control for the mathematical impact of heart rate (HR) on HRV (Sacha, 2014), mean heart rate was extracted and used as a controlling variable in the generalized models.

root mean square of successive differences (RMSSD)

RMSSD is a much-used measure of vagally-mediated high-frequency heart rate variability (HF-HRV) that is highly correlated with high-frequency components of the heart signal (Massin et al., 1999). To ascertain the validity of this assumption in the current data set, RMSSD was correlated against a measure of absolute HF power in the RR-signal (HF power ms^2, calculated by autoregressive modeling, 16 model-order) yielding a very strong positive relationship between RMSSD and HF ms^2 in both groups (clinical $r = 0.94$, $p \leq 0.001$; control $r = 0.95$, $p \leq 0.001$).

Calculation of beat-to-beat instantaneous HR and RMSSD

To allow for investigation of possible group, age or gender distinctions in the development of HR and RMSSD across the 5 min of baseline, instantaneous beat-to-beat HR and RMSSD was calculated from the interbeat-interval in R (R Core Team, 2017) using the "varian" package (Wiley and Elkhart Group Limited, 2016). RMSSD calculated from ultrashort 30-s segments have shown good correspondence with longer recordings (Munoz et al., 2015), so 30-s segments were chosen. For each 30-s interval the mean HR and RMSSD for each subject was calculated.

sample entropy (SampEn)

SampEn (Richman and Moorman, 2000; Lake et al., 2002) calculates the conditional probability that two pattern sequences of m number of consecutive points that match within a tolerance r, still match when one additional consecutive point is included. High values of SampEn indicate high irregularity and complexity in the signal. SampEn was calculated with a tolerance (r) of 0.2 standard deviation of the R-R interval and an embedding dimension (m) of 2.

detrended fluctuation analysis (DFA)

DFA was calculated on the original RR-interval time-series before the detrending filter was applied to avoid detrending twice. The DFA algorithm is a modified root mean square analysis of a random walk (Peng et al., 1995). DFA is used to detect long-term correlations in a non-stationary time series, and calculates the correlation within non-overlapping segments of the signal. In each segment a least-squares regression-line is formed, and a detrended time series is then generated by subtracting regression from the integrated series. The final fluctuation is calculated from the detrended and integrated series, giving a scaling exponent, α. For this study the short term scaling exponent, DFA-α_1, was calculated with n varying from 4 to 16 beats, giving an estimate of short-term correlations in the signal. Within these parameters DFA-α_1 will likely capture a mixture of both low- and high-frequency components. A short term scaling exponent of α_1 of 0.5 is indicative of a random and uncorrelated signal resembling white noise while an α_1 level between 0.5 and 1 is indicative of positive correlations and self-similarity in the signal. Conversely, a signal between 0 and 0.5 is indicative of negative correlations in the signal (Hardstone et al., 2012).

pre-ejection period (PEP)

PEP was analyzed in Mindware IMP 3.1.4, using the baseline impedance signal (Z_0) and rate of change in impedance (dZ/dt). The signal was inspected visually for noise and noisy segments were excluded. PEP was identified based on the interval separating the R-onset/Q-peak in the ECG, and the B-point in the dZ/dt signal. The R/Q point was estimated using minimum value K-R interval (K value 35) (Berntson et al., 2004) and the B-point was estimated through percent of dZ/dt time (55 percent) (Lozano et al., 2007). An average PEP for five 60-s epochs was calculated using a scale factor of 0.09 V/Ω for Z_0 and 1.0 V/Ω for dZ/dt.

Statistical Analysis

For statistical analysis SPSS version 25 (IBM) and R version 3.4.4 (R Project) was employed. Outliers in the psychophysiological and psychological data (± 3.29 SD) were removed. Between group-differences on the most important variables were checked with independent t-tests. Differences in the instantaneous HR and RMSSD were investigated using t-tests on individual means within each non-overlapping 30-s subinterval of the 5-min recording. Analysis was run checking for differences resulting from group-membership, age (by median split at 10.7 years) and sex.

Effect size for significant between group-differences is given as Cohen's d. The correlations between cardiac variables and the relationship between the non-linear HRV variables and the EATQ-R subscales were checked with Pearson product moment correlations. The Affective Problems and Anxiety Problems subscales were analyzed with non-parametric Spearman's rank-order correlations, due to a distribution skewed toward low problems in the control group. Strengths of associations are discussed as follows: a correlation coefficient of 0.30 to 0.50 indicates a weak relationship, 0.50 to 0.70 a moderate

relationship, 0.70 to 0.90 a strong relationship and > 0.90 indicates a very strong relationship between variables.

Generalized linear modeling with a maximum likelihood estimate and link ID function was used for analysis of the relationships between the physiological variables and internalizing psychopathology and negative affect. Results are given as full model effects for all variables added to each model. For variables showing significance above 0.01 and 0.05 level, B (and 95 % confidence intervals) and Exp(B) are also given. The B-value represents the slope in the linear equation, and can be read as follows: when X (the independent variable) rises with 1, this will lead to an increase/decrease in Y (the dependent variable) that equals B. All independent variables were transformed to Z-scores for a more standardized interpretation of the B-values across variables. Exp(B) is an odds-ratio estimate of an outcome given a one unit increase in the independent variable, and gives an estimate of effect size. An Exp(B) above 1 indicates a likely increase in the dependent variable given an increase in the independent variable, while an Exp(B) of less than 1 indicates a likely decrease in the dependent variable given an increase in the independent variable.

Significance will be discussed at both a $p \leq 0.01$ and a $p \leq 0.05$ level to balance the risk of type 1 and type 2 errors due to sample size and the exploratory nature of the study. The results need to be interpreted with this in mind.

RESULTS

Between-Group Differences

The clinical and control group differed significantly on the Internalizing Problems, Negative Affect and Effortful Control scales. The two groups did not differ significantly in regard to SampEn, DFA-α_1, HR or RMSSD for the five-minute sample as a whole, but PEP was shorter in the control group than in the clinical group. Descriptive statistics and between group differences can be seen in **Table 1**. Analysis of instantaneous HR and RMSSD in 30-s segments across the 5 min showed no significant differences between the clinical subjects and the controls, males and females or younger and older subjects.

Correlations Between HRV-Variables

SampEn showed a weak negative correlation to HR in the control group, and no significant relationship to any other cardiac variables. DFA-α_1 was moderately positively related to HR in the clinical group and moderately negatively related to RMSSD in both groups. RMSSD was also moderately negatively related to HR in both groups, while PEP showed a weak relationship to HR (negative) and RMSSD (positive) in the control group. See **Tables 2**, **3** for results.

Correlations With the CBCL and EATQ-R-Subscales

Further, we ran correlations between the non-linear cardiac variables and the CBCL subscales of Affective and Anxiety Problems. SampEn was weakly negatively related to Affective Problems at a $\leq$ 0.05 level and showed a nearly-significant trend toward a weak negative relationship with Anxiety Problems in the clinical group. For the control group there was a moderate negative correlation with Anxiety Problems at a $\leq$ 0.01 significance level. DFA-α_1 did not show any significant relationships in either group. See **Table 4** for results.

TABLE 1 | Descriptive statistics and between-group differences.

	Clinical group			Control group			Between-group differences		
	n	*Mean*	*SD*	*n*	*Mean*	*SD*	*t*	*p*	*d*
Internalizing Prob.	32	6.4	0.8	25	4.4	0.8	9.1	**≤0.001*** **	2.4
Affective Problems	32	6.4	0.9	25	5.1	0.3			
Anxiety Problems	32	6.4	0.7	25	5.1	0.3			
Negative Affect	32	2.7	0.5	25	2.0	0.5	4.8	**≤ 0.001*** **	1.3
Aggression	32	2.2	0.7	25	1.7	0.6			
Frustration	32	2.9	0.8	25	2.4	0.7			
Depressive Mood	32	3.1	0.7	25	2.0	0.6			
Fear	32	2.6	0.6	25	2.1	0.7			
Shyness	32	2.9	0.9	25	2.3	0.6			
Effortful Control	32	3.4	0.6	25	3.8	0.5	−2.5	**0.018***	−0.7
HR	32	78.2	8.3	25	80.3	8.8	−0.9	0.36	
PEP	30	122.3	6.1	25	117.9	5.8	2.7	**0.009****	0.7
RMSSD	31	73.8	39.4	25	67.9	35.9	0.6	0.56	
SampEn	31	1.7	0.2	25	1.7	0.2	0.4	0.71	
DFA-α_1	32	0.8	0.2	25	0.8	0.2	−0.3	0.77	

*Between group differences was conducted using independent t-tests (t); SD, standard deviation; DFA, detrended fluctuation analysis; HR, mean heart rate; RMSSD, root mean square of successive differences; PEP, pre-ejection period; SampEn, sample entropy; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, a minor portion of this table has been published in Fiskum et al. (2017)*

TABLE 2 | Correlations between cardiac variables in the clinical group.

		MeanHR	PEP	RMSSD	SampEn
MeanHR	*r*	1			
	p	–			
	n	32			
PEP	*r*	−0.32	1		
	p	0.08	–		
	n	30	30		
RMSSD	*r*	**−0.61*****	0.15	1	
	p	<0.001	0.43	–	
	n	31	29	31	
SampEn	*r*	−0.19	0.16	−0.25	1
	p	0.31	0.40	0.18	–
	n	31	29	30	31
DFA-α_1	*r*	**0.57*****	−0.29	**−0.65*****	0.19
	p	0.001	0.13	<0.001	0.31
	n	32	30	31	31

*r, Pearson product-moment correlation coefficient; DFA, detrended fluctuation analysis; RMSSD, root mean square of successive differences; PEP, pre-ejection period; SampEn, sample entropy; ***p ≤ 0.001.*

TABLE 3 | Correlations between cardiac variables in the control group.

		MeanHR	PEP	RMSSD	SampEn
MeanHR	*r*	1			
	p	–			
	n	25			
PEP	*r*	**−0.47***	1		
	p	0.02	–		
	n	25	25		
RMSSD	*r*	**−0.68*****	**0.49****	1	
	p	<0.001	0.01	–	
	n	25	25	25	
SampEn	*r*	**−0.46***	0.04	0.15	1
	p	0.02	0.87	0.47	–
	n	25	25	25	25
DFA-α_1	*r*	0.35	−0.33	**−0.53****	−0.31
	p	0.09	0.10	0.01	0.13
	n	25	25	25	25

*r, Pearson product-moment correlation coefficient; DFA, detrended fluctuation analysis; RMSSD, root mean square of successive differences; PEP, pre-ejection period; SampEn, sample entropy; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.*

We also ran correlations between the non-linear cardiac variables and the EATQ-R Negative Affect subscales (Aggression, Fear, Frustration, Depression and Shyness) as well as the Effortful Control scale. For the EATQ-R scales a $\leq$ 0.01 level significant negative relationship of moderate strength could be found between SampEn and the subscales Aggression, and Frustration in the clinical group. There was a weak positive relationship between SampEn and Effortful Control ($p \leq 0.05$). There were no significant correlations in the control group. DFA-α_1 was not significantly related to any of the subscales in either group. See **Table 5** for results.

TABLE 4 | Correlations between CBCL clinical scales and cardiac variables.

		Affective Problems	Anxiety Problems
CLINICAL GROUP			
SampEn	ρ	**−0.41***	−0.35
	p	0.02	0.053
	n	31	31
DFA-α_1	ρ	−0.16	−0.19
	p	0.38	0.31
	n	32	32
Control Group			
SampEn	ρ	0.06	**−0.50****
	p	0.79	0.01
	n	25	25
DFA-α_1	ρ	0.13	0.09
	p	0.55	0.68
	n	25	25

*ρ, Spearman's ranked order correlation coefficient; DFA, detrended fluctuation analysis; SampEn, sample entropy; *p ≤ 0.05; **p ≤ 0.01.*

SampEn, DFA-α_1, RMSSD and PEP as Discriminators of Internalizing Psychopathology

We ran two generalized linear models with Internalizing Problems as the dependent variable, and SampEn (Model 1) and DFA-α_1 (Model 2) as independent variables alongside RMSSD and PEP. All models also controlled for group, sex, age, BMI, HR and a group-interaction. Both models were significant, with no significant group-interactions.

The results from Model 1 showed that a higher SampEn was significantly associated with less psychopathology at a $\leq$ 0.01 confidence level. RMSSD and PEP did not reach significance.

The results from Model 2 showed that a higher value of DFA-α_1 was related to less Internalizing Problems at a $\leq$ 0.05 confidence level. RMSSD and PEP were not significantly related to Internalizing Problems.

Model effects and effect sizes for both models can be seen in **Table 6**.

SampEn, DFA-α_1, RMSSD and PEP as Discriminators of Negative Affect

We ran two generalized linear models with Negative Affect as the dependent variable and SampEn (Model 3) and DFA-α_1 (Model 4) as independent variables alongside RMSSD and PEP. All models also controlled for group, sex, age, BMI, HR and a group-interaction. Both models were significant, with no significant group-interactions.

The results from Model 3 showed that a higher value of SampEn was significantly related to less Negative Affect at a $\leq$ 0.01 level. RMSSD also reached significance in this model ($p \leq 0.05$), with greater RMSSD associated with less Negative Affect.

For Model 4 DFA-α_1 showed a trend toward significance at a $\leq$ 0.05 level, with a higher value being related to less Negative

TABLE 5 | Correlations between EATQ-R scales and cardiac variables.

		Aggression	Frustration	Depressive Mood	Fear	Shyness	Effortful Control
CLINICAL GROUP							
SampEn	*r*	**−0.53****	**−0.52****	−0.30	−0.28	−0.19	**0.40***
	p	0.002	0.003	0.11	0.13	0.31	0.02
	n	31	31	31	31	31	31
DFA-α_1	*r*	−0.09	−0.21	−0.11	−0.17	−0.03	−0.08
	p	0.61	0.25	0.57	0.36	0.88	0.67
	n	32	32	32	32	32	32
CONTROL GROUP							
SampEn	*r*	−0.18	−0.36	−0.04	−0.32	−0.15	0.13
	p	0.39	0.08	0.84	0.12	0.48	0.55
	n	25	25	25	25	25	25
DFA-α_1	*r*	0.36	−0.01	−0.02	−0.22	0.22	0.004
	p	0.08	0.95	0.93	0.28	0.28	0.99
	n	25	25	25	25	25	25

*r, Pearson product-moment correlation coefficient; DFA, detrended fluctuation analysis; SampEn, sample entropy; *p ≤ 0.05; **p ≤ 0.01.*

TABLE 6 | Model effects for DFA-α_1, SampEn, RMSSD, and PEP as predictors of internalizing psychopathology.

						95% CI				
		n	***df***	χ^2	p_{χ^2}	**−95%**	**B**	**+95%**	**EXP(B)**	p_B
Model effects for SampEn, RMSSD, and PEP as predictors of Internalizing Psychopathology.										
Full model		52	9	55.2	**≤ 0.001*****					
Factors	Group			56.9	**≤ 0.001*****	−2.4	−1.9	−1.4	0.2	≤ 0.001
	Sex			0	0.97					
Co-variables	Age			0.1	0.72					
	BMI			0.8	0.39					
	HR			0.6	0.46					
Predictors	RMSSD			1.5	0.23					
	PEP			0.0	0.93					
	SampEn			8.0	**0.005****	−0.8	−0.4	−0.1	0.7	0.024
Interaction	Group × SampEn			0.3	0.56					
Model effects for DFA-α_1, RMSSD, and PEP as predictors of Internalizing Psychopathology										
Full model		54	9	57.0	**≤ 0.001*****					
Factors	Group			80.8	**≤ 0.001*****	−2.6	−2.1	−1.7	0.1	≤ 0.001
	Sex			1.3	0.26					
Co-variables	Age			0.3	0.56					
	BMI			0.0	0.99					
	HR			0.7	0.41					
Predictors	PEP			0.5	0.49					
	RMSSD			0.5	0.48					
	DFA-α_1			4.3	**0.039***	−0.9	−0.5	−0.1	0.6	0.010
Interaction	Group × DFA-α_1			2.9	0.09					

*Results were calculated with a generalized linear model using a normal probability distribution and identity link function. BMI, body mass index; HR, heart-rate; RMSSD, root mean square of successive differences; PEP, pre-ejection period; SampEn, sample entropy; DFA, detrended fluctuation analysis; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.*

TABLE 7 | Model effects for DFA-α_1, SampEn, RMSSD, and PEP as predictors of Negative Affect.

Model effects for SampEn, RMSSD, and PEP as predictors of Negative Affect										
						95% CI				
		n	***df***	χ^2	p_{χ^2}	**−95%**	**B**	**+95%**	**EXP(B)**	p_B
Full model		52	9	37.9	**≤ 0.001*** **					
Factors	Group			34.1	**≤ 0.001*** **	−1.0	−0.7	−0.5	0.5	≤ 0.001
	Sex			0.6	0.46					
Co-variables	Age			2.2	0.14					
	BMI			1.9	0.17					
	HR			1.6	0.21					
Predictors	RMSSD			5.3	**0.021***	−0.5	−0.3	−0.04	0.8	0.021
	PEP			1.5	0.22					
	SampEn			8.9	**0.003****	−0.5	−0.3	−0.1	0.8	0.005
Interaction	Group × SampEn			0.1	0.77					
Model effects for DFA-α_1, RMSSD, and PEP as predictors of Negative Affect										
Full model		53	9	34.6	**≤ 0.001*** **					
Factors	Group			39.8	**≤ 0.001*** **	−1.0	−0.8	−0.6	0.5	≤ 0.001
	Sex			3.2	0.074					
Co-variables	Age			1.6	0.21					
	BMI			0.0	0.98					
	HR			0.5	0.50					
Predictors	PEP			4.0	**0.045***	−0.3	−0.2	−0.003	0.9	0.045
	RMSSD			1.5	0.22					
	DFA-α_1			3.1	0.08					
Interaction	Group × DFA-α_1			1.8	0.18					

*Results were calculated with a generalized linear model using a normal probability distribution and identity link function. BMI, body mass index; HR, heart-rate; RMSSD, root mean square of successive differences; PEP, pre-ejection period; SampEn, sample entropy; DFA, detrended fluctuation analysis; *p≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.*

Affect. PEP reached significance ($p \leq 0.05$), with a longer PEP associated with less Negative Affect.

Model effects and effect sizes for both models can be seen in **Table 7**.

DISCUSSION

Non-linear HRV, Internalizing Psychopathology and Negative Affect

Several studies have shown that internalizing psychopathology and dysregulated negative affect are associated with a loss of non-linear HRV in the heartbeat in adults (Kemp et al., 2010; de la Torre-Luque et al., 2016), but the relationship between non-linear HRV and psychopathology is not as well studied in children (Paniccia et al., 2017). Two main domains of methods have shown promise, the informational domain, including sample entropy (SampEn), and the invariant domain, including detrended fluctuation analysis (DFA). This study investigated SampEn and DFA-α_1 in children related to internalizing psychopathology and negative affect. As HRV has been proposed as a biomarker of dysregulation and psychopathology (Beauchaine and Thayer, 2015; Kemp et al., 2017), and the neurovisceral integration model (Thayer and Lane, 2000, 2009) models psychopathology as a loss of vagal tone and resulting disinhibition of the SNS, we also wanted to see if non-linear methods could provide more information than a measure of vagally-mediated high-frequency HRV, the root mean square of successive differences (RMSSD) and a measure of SNS-mediated cardiac contractility, pre-ejection period (PEP).

The results showed that a higher value of SampEn was significantly associated with less internalizing psychopathology and negative affect when modeled alongside RMSSD and PEP. A higher DFA-α_1 was also significantly related to less internalizing psychopathology, and showed a trend toward significance for negative affect, in the same direction. Neither RMSSD nor PEP were significantly related to internalizing psychopathology. Higher RMSSD showed a significant relationship to less negative affect when modeled alongside SampEn. A longer PEP, indicating less SNS activity, was related to less negative affect when modeled alongside DFA-α_1. Correlational analysis between SampEn and DFA-α_1 and the EATQ-R subscales related to internalizing psychopathology and negative affect showed that having a higher SampEn was linked to less aggression and frustration in the clinical group. There was also a positive relationship between higher SampEn and better effortful control, a protective factor against psychopathology linked to frontal function, in the clinical group. This links SampEn to established indices of frontal function and dysfunction (Bishop et al., 2004; Blair, 2004; Fekete et al., 2014). DFA-α_1 was

not significantly related to any of the subscales in either group.

Between-Group Differences

There were no significant differences between the two groups in HR, RMSSD, SampEn or DFA-α_1 for the 5-min recordings. The clinical group showed lower sympathetic activation, as reflected in a longer PEP, than the control group. In the neurovisceral integration model psychopathology is linked to increased SNS activation, so these results are contradictory to both theory (Thayer and Lane, 2000, 2009) and previous empirical findings linking shorter PEP (Light et al., 1998; Schweiger et al., 1998; Buss et al., 2004; Muñoz and Anastassiou-Hadjicharalambous, 2011) to internalizing problems in children and adults.

The lack of significant differences for the non-linear variables could mean that neither SampEn nor DFA-α_1 may be sensitive enough to distinguish between subjects suffering from less-severe psychopathology and normal-functioning controls. The clinical group was not a high-psychopathology group, instead presenting with lighter clinical problems related to anxiety and depression. Following this there might have been differences between the control group and a more pathological group. The lack of difference in RMSSD is supportive of this hypothesis, as resting state recordings of RMSSD have been found to distinguish between healthy controls and children and adolescents with clinical problems (Koenig et al., 2016; Paniccia et al., 2017). It is also possible that the situation itself, which was a resting baseline without a specific stressor, was not sufficiently powerful to elicit reliable group differences related to autonomic stress responsivity. A reactive laboratory task or an ecological measurement of stressful activation in the environment would likely be able to yield more information on group differences. A more finely grained analysis of the non-linear measures as they evolved over a time-line might also have yielded further results.

Another important consideration is tied to the possibility of differences in multilevel complexity. Non-linear complexity, or loss thereof, may reveal itself on different timescales (Bornas et al., 2006), making a case for a multi-scaled analysis of non-linear complexity (Costa et al., 2002). A further multi-scale entropy analysis of the signal on different time scales could have revealed more information, and the same could have been the case for further investigation of different scales of resolution for DFA-α_1, but this was beyond the scope of the current study.

The Nature of the Relationship Between Cardiac Complexity, Internalizing Psychopathology and Negative Affect: The Optimum Variability Hypothesis

Taken together, the results from the generalized models and correlations showed a linear relationship between SampEn and internalizing psychopathology and negative affect. The correlational analyses were mostly significant in the clinical group, but the relationships were generally in the same direction across groups. The clinical sample showed higher levels of internalizing psychopathology and negative affect, which could account for the discrepancies in the strength of the associations in the correlations. A recent meta-analysis of the correspondence of vagally-mediated HRV measures to depressive problems in children also found non-significant correlational results for healthy controls (Koenig et al., 2016), indicating that the relationships may be less pronounced in healthy populations. Another possibility is that the relationship is quadratic, rather than linear.

It has been assumed that higher variability or complexity is linearly related to health (Guastello, 2015), and this has generally been supported by empirical findings related to psychopathology (de la Torre-Luque et al., 2016). However, the hypothesis of optimal variability posits a quadratic relationship, where optimal function resides in a band of optimal complexity, defined by high organization and high openness to input-driven variability (Guastello, 2015; Schuldberg, 2015). Outside of the range of optimal variability function will be affected negatively, due to either too much organizational constraint or too little stability. This quadratic relationship may present as linear in smaller samples, or in samples spanning the lightly-dysfunctional to the normally-functional. If this happens, relationships in the data may present as smaller than they would be, given a larger or more heterogenous population (Guastello, 2015). In this study, the relationship between non-linear HRV and internalizing psychopathology and negative affect presented as linear, with higher SampEn associated with less internalizing psychopathology and less negative affect and a higher DFA-α_1 linked to less internalizing psychopathology. There was a stronger relationship in the clinical group than in the healthy controls, although group-status did not yield a significant interaction in any of the models. Following the optimal variability hypothesis, it could be that a larger, or differently-distributed sample could have revealed a different and perhaps more curvilinear relationship between the non-linear HRV-variables and psychopathology and negative affect, and that the weaker relationship in the healthy sample is indicative of a relationship that may actually be curvilinear in the population (Guastello, 2015).

Implication of the Results

In the neurovisceral integration model (Thayer and Lane, 2000, 2009) negative affect and psychopathology can be modeled as a loss of adaptability in the neurovisceral system characterized by a state of chronic frontal disinhibition and vagal withdrawal. This dysregulated state in turn leads to sympathetic hyperarousal and negative attentional, emotional, cognitive, behavioral and physiological consequences. The results in this study support that lower vagal and higher SNS activation were related to dysregulation of negative affect, although the clinical group showed lower SNS activation as indexed by PEP than the controls. The results also indicated that vagal and SNS activation were not the entire story, as the generalized models showed that SampEn and DFA-α_1 were more sensitive indicators of internalizing psychopathology than vagally-mediated HF-HRV and sympathetic activation combined. SampEn also held explanatory power beyond that accounted for by RMSSD and PEP related to negative affect. This indicates that non-linear HRV can provide information beyond what is contained in linear

indices of vagal and SNS activation. It is however harder to pinpoint what non-linear HRV indexes, in terms of physiological correlates.

To try to clarify this question we have to distinguish between the physiology underlying informational complexity, as indexed by SampEn, and the physiology potentially involved in invariant short term self-similarity, as indexed by DFA-α_1. Both informational and short-term invariant complexity have been found to be comparable between children and young adults, with a decline in informational complexity after age 40 (Pikkujämsä et al., 1999). DFA-α_1 has been shown to be unrelated to sympathetic norepinephrine spillover, a marker of sympathetic cardiac activation (Baumert et al., 2009). DFA-α_1 has also been shown to be negatively related to RMSSD, and to increase after vagal blockade (Perkiomaki et al., 2002). In our study DFA-α_1 was strongly negatively related to RMSSD, and showed no relation to PEP. This indicates a likely vagal influence on DFA-α_1 in our study.

In regard to the potential functional significance of DFA-α_1, one hypothesis is that invariant self-similarity in a signal represents a form of internal long-term "memory" of a system (West, 2014). While a simple thermostat with one set-point reacts only to real-time fluctuations in temperature, with no memory of earlier fluctuations, this is not the case for complex systems. Behavior in a complex system relates to multiple set-points, across different time-scales. As a consequence, instead of operating only on one time-scale, the system displays multi-stability across different time-scales. In an earlier publication one of the present authors investigated a link between the attentional orienting network, vagal control and non-linear scaling properties (Balle et al., 2015). In this study, deficits in the attentional network were accompanied by alterations in the scaling properties, or long-term "memory" of the cardiac system.

The self-similar properties of the cardiac system may also be relevant in relation to interoception (Vaitl, 1996), where bodily signals, through for instance vagally-mediated afferent baroreceptor firing (Garfinkel et al., 2013, 2015), represent an internal context important for perception (Dunn et al., 2010; Seth, 2013). It has been shown that the perception of frightening emotional stimulus can be enhanced at different points in the cardiac cycle related to systole and diastole (Garfinkel et al., 2014; Garfinkel and Critchley, 2016). It has also been shown that high accuracy in interoception facilitates the predictability of internal signals, possibly increasing resilience to the effect of stimulus presentation timing (Garfinkel et al., 2013). Furthermore, the inability to integrate and predictively utilize information from interoception may be a common factor underlying emotional dysregulation and anxiety (Seth, 2013), linked to the anterior cingulate cortex and the anterior insula, two areas that are important for a feeling of "self-in-context" (Seth et al., 2012; Seth, 2013). In our study a higher DFA-α_1 was related to less internalizing psychopathology. It could be speculated that perhaps higher scale-free self-similarity in the cardiac signal not only indexes a more complex underlying organization of the neurocardiac system, but also represents a more predictable inner context, enhancing the potential for predictability in interoception. Following this, perhaps a higher potential for predictability in inner context can have a positive effect on emotional perception and processing and the experience of "self-in-context" relevant for mental health. This would need to be investigated further and remains purely hypothetical at this point.

With respect to the physiological correlates of SampEn, the informational complexity in a signal is thought to reflect on the complexity and flexibility of the underlying system (Bertuglia and Vaio, 2005). A low-entropy system behaves in a very repetitive manner (the rate of new information it generates is close to zero), and is therefore less capable of adapting to the changing environment than a high-entropy system. Healthy hearts do not beat at a totally constant rate, but show many fluctuations that make it difficult to predict the length of the next interbeat interval. At the same time this increases their adaptability. Reduced cardiac entropy has been found to be related to vagal withdrawal and increased SNS-activation in anxious patients (see for instance de la Torre-Luque et al., 2016), with some exceptions linked to higher irregularity (and hence entropy) of respiration in panic-disordered patients (see for instance Caldirola et al., 2004). Evidence from vagal blockade (Millar et al., 2010) and norepinephrine spillover (Baumert et al., 2009) point to a likely higher influence of vagal than sympathetic activation in entropy rate. At the same time, investigation has also failed to find an effect of vagal blockade along with no correlations between entropy and cardiac measures of vagal origin (Perkiomaki et al., 2002). In our study SampEn was not significantly correlated with either RMSSD or PEP, but showed a weak negative relationship to heart rate in the controls. When RMSSD and PEP were modeled together as independent variables affecting SampEn, (as reported in Fiskum et al., 2017), lower RMSSD and higher PEP were linked to greater entropy. This indicates that SampEn did not relate to either vagal or SNS influence in a simple one-to-one linear way. From a theoretical point of view, non-linear cardiac complexity should indeed reflect on more emergent characteristics of whole-system behavior, than simpler, linear metrics (Bertuglia and Vaio, 2005; Tyukin, 2011; West, 2014).

From a more functional perspective, reduced SampEn has been linked to reduced flexibility in the neurovisceral system in adolescents at risk for anxiety disorder (Balle et al., 2013). A hypothesis in line with this is that non-linear HRV represents an index of the flexibility of the organization of the neurocardiac system, linked to underlying brain connectivity (Young and Benton, 2015). It is interesting to note that HF-HRV has been linked to higher local connectivity in the perigenual anterior cingulate cortex, a medial prefrontal area serving as a connective hub between the salience and default mode networks (Jennings et al., 2016). Resting HF-HRV has also been linked to stronger functional connectivity between amygdala and the medial-prefrontal cortex (Sakaki et al., 2016). The analysis of the subscales in this study linked levels of SampEn with markers of frontal and limbic function. Anxiety, aggression and frustration have been linked to diminished frontal functionality (Bishop et al., 2004; Blair, 2004) and greater amygdala reactivity

(Rauch et al., 2003), while better effortful control has been linked to greater frontal functionality and connectivity (Fekete et al., 2014). Taken together, the results in this study show that SampEn may have potential as an index of the organizational flexibility and adaptability of the neurovisceral system, relevant to psychopathology and negative affect, that in turn may be linked to the organization of frontal and limbic areas of the brain.

CONCLUSION

Overall, the results from this study were in line with the neurovisceral integration model in that lower SNS and higher vagal activation were related to less dysregulated negative affect. In line with the initial hypotheses information-based complexity, as indexed by SampEn, explained variation in negative affect beyond the combination of PNS/SNS activation. The results also showed that SampEn and DFA-α_1 were the only variables significantly related to internalizing psychopathology, apart from group status.

This means that non-linear HRV provided information about the operation of the neurovisceral system relevant to internalizing psychopathology and negative affect beyond that of indices of parasympathetic and sympathetic activation combined. It has been suggested that HRV can be a measure of autonomic flexibility (Lipsitz, 2004; Beauchaine and Thayer, 2015), with low variability indexing a rigid autonomic balance, that is, a system that is less capable of flexible interaction with the outside environment. We propose that non-linear HRV holds potential as a complementary way to index adaptability related to the organization of the underlying neurovisceral system. Specifically, we suggest that cardiac entropy may be an indicator of the flexibility of the neurovisceral system underlying emotional regulation and adaptive behavior, that may be linked to integrated functionality or connectivity in frontal and limbic areas. We also propose a hypothesis that invariant self-similarity may influence a form of bottom-up predictability involved in interoception (or inner context), that may serve as a protective influence in regard to psychopathology.

Limitations and Future Research

The sample size was small, and this means that small- and moderate-effect-size relationships may have been overlooked (Quintana, 2017). Also, this study focused on internalizing psychopathology and negative affect, and did not investigate disorder across the externalizing or thought-disorder spectrum, which may have yielded further results. Another limitation is that the clinical group was not a high-psychopathology group. The clinical subjects were referred for lighter problems of anxiety and depression, meaning that the results may have presented differently in a more pathological group. The age-distribution of the sample also meant that the children may have been in different stages of puberty. Non-linear HRV in children between 6 and 15 years have been shown to not differ significantly from young adults, meaning that the measures of non-linear HRV should still be valid (Pikkujämsä et al., 1999). A further limitation is the lack of child-report of psychopathology and negative affect. To make up for this, the parent-report tools used were chosen for extensive clinical experience and use. The CBCL has also shown good correspondence between parent ratings and clinical judgment of psychopathology by professionals (Verhulst and van der Ende, 1991) and diagnostic criteria derived from the Diagnostic and Statistical Manual of Mental Disorders (DSMuncorrelated signal resembling) (Ebesutani et al., 2010).

Furthermore, it is a limitation that no autonomic stress maneuver designed to elicit sympathetic activation or a measure of sympathetic variability was employed. PEP can be influenced by several cardiovascular confounders including left ventricular end-diastolic pressure, systemic vascular resistance and diastolic blood pressure (Krohova et al., 2017), and none of these factors were controlled for. The inclusion of QT-variability, an index of spontaneous beat-to-beat fluctuations in the QT-interval indexing cardiac repolarization lability (Baumert et al., 2016), would have strengthened the results. QT-variability has been linked to internalizing spectrum psychopathology (Yeragani et al., 2003; Koschke et al., 2009), also alongside differences in non-linear HRV (Yeragani et al., 2001) and should be considered as a focal measure of sympathetic variability in future studies. A suitable stress maneuver should also be included. The lack of control for respiration is a further limitation, as respiratory patterns can affect HRV.

Finally, the physiological correlates of non-linear HRV are not easy to pin down, and the relationships to psychopathology and health are not necessarily straightforward, so more research remains to show the clinical usefulness. While much research on HRV and emotional dysregulation is based on the analysis of resting baseline HRV-data, studies looking at non-linear HRV-data during the completion of stressful or negatively loaded emotional tasks would likely give additional information about the relationship between complexity, state and dysregulation. The results should be further explored in both younger and older populations, in community-based samples, and in other clinical populations as there may be differences across age- and patient-groups. One line of investigation that could potentially increase clinical usefulness would be the study of non-linear HRV-indices related to characteristics of brain-network connectedness in the neurovisceral system. Also, further research into invariant cardiac self-similarity as a potential moderator of the predictability of interoception, and possible effects on psychopathology and emotional dysregulation should be conducted.

AUTHOR CONTRIBUTIONS

CF contributed to the original project formulation, conducted data collection, linear and non-linear HRV-analysis, statistical analysis and wrote the paper. TA contributed to the original project formulation, conducted data collection, PEP analysis, contributed on linear HRV-analysis and critically reviewed the paper. XB supervised and gave input on non-linear analysis and critically reviewed the paper. PA supervised and gave input on statistical analysis and critically reviewed the paper. KJ and MF

supervised and contributed on the original project formulation and critically reviewed the paper.

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Linear and Nonlinear Analyses of the Cardiac Autonomic Control in Children With Developmental Coordination Disorder: A Case-Control Study

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Children with Developmental Coordination Disorder (DCD) and children at risk for DCD (r-DCD) present motor impairments interfering in their school, leisure and daily activities. In addition, these children may have abnormalities in their cardiac autonomic control, which together with their motor impairments, restrict their health and functionality. Therefore, this study aimed to assess the cardiac autonomic control, by linear and nonlinear analysis, at supine and during an orthostatic stimulus in DCD, r-DCD and typically developed children. Thirteen DCD children (11 boys and 2 girls, aged 8.08 ± 0.79 years), 19 children at risk for DCD (13 boys and 6 girls, aged 8.10 ± 0.96 years) and 18 typically developed children, who constituted the control group (CG) (10 boys and 8 girls, aged 8.50 ± 0.96 years) underwent a heart rate variability (HRV) examination. R-R intervals were recorded in order to assess the cardiac autonomic control using a validated HR monitor. HRV was analyzed by linear and nonlinear methods and compared between r-DCD, DCD, and CG. The DCD group presented blunted cardiac autonomic adjustment to the orthostatic stimulus, which was not observed in r-DCD and CG. Regarding nonlinear analysis of HRV, the DCD group presented lower parasympathetic modulation in the supine position compared to the r-DCD and CG groups. In the within group analysis, only the DCD group did not increase HR from supine to standing posture. Symbolic analysis revealed a significant decrease in 2LV ($p < 0.0001$) and 2UV ($p < 0.0001$) indices from supine to orthostatic posture only in the CG. In conclusion, r-DCD and DCD children present cardiac autonomic dysfunction characterized by higher sympathetic, lower parasympathetic and lower complexity of cardiac autonomic control in the supine position, as well as a blunted autonomic adjustment to the orthostatic stimulus. Therefore, cardiovascular health improvement should be part of DCD children's management, even in cases of less severe motor impairment.

Keywords: heart rate variability, autonomic nervous system, developmental coordination disorder, autonomic dysfunction, orthostatic stimulus, motor impairment

INTRODUCTION

Developmental Coordination Disorder (DCD) is a disorder of motor coordination that significantly impairs the motor actions in children in different age groups (Harris et al., 2015). Current diagnostic guidelines for DCD involve a continuum of factors that address motor impairments in different children's life context (Blank et al., 2012; American Psychiatric Association, 2013). Due the complexity and difficulty in diagnosis, DCD prevalence around the world has varied from 1.7% (Lingam et al., 2009) to 24% (Valentini et al., 2017) among children of school age. Moreover, in addition to the terminology "children with DCD," the term "children at risk for DCD" is also used in the literature, corresponding to the profile of children that present a motor impairment condition, but occupy the intermediate classification in standard motor tests (Smits-Engelsman et al., 2015; Wilson et al., 2017).

DCD related motor impairments contribute to restricting children's engagement with tasks involving accuracy and speed of movement (Licari et al., 2015; Wilson et al., 2017), which might cause fear of frustration and/or embarrassment (Cummins et al., 2005). Therefore, these children are more likely to choose solitary tasks and with more sedentary characteristics (Sylvestre et al., 2013). As a consequence, children with DCD have lower levels of habitual physical activity (Hendrix et al., 2014) tending to develop overweight and obesity (Cermak et al., 2015), thus increasing the risk for developing cardiovascular diseases (Rivilis et al., 2011).

Regarding cardiovascular risk assessment, the use of heart rate variability (HRV) analysis has been extensively applied to study the cardiac autonomic control in different populations and conditions, since it is a low-cost noninvasive tool providing important prognostic parameters for cardiovascular mortality, even in individuals without previous cardiovascular pathologies (Hillebrand et al., 2013; Wulsin et al., 2015).

Interestingly, some studies have reported autonomic dysfunction in subjects with motor disabilities (Hamamoto et al., 2003; Zamunér et al., 2011). However, studies assessing cardiac autonomic control in DCD are incipient. Coverdale et al. (2012) studied the cardiac autonomic control and baroreflex sensitivity in the resting supine condition in adolescents with DCD. The authors reported no differences between adolescents with probable DCD and healthy controls regarding linear HRV indices, but reported reduced baroreflex sensitivity, which was mainly attributed to an increased percentage of body fat. Chen et al. (2015) sought to study HRV in children at risk for DCD during cognitive tasks and concluded that they may show decreased HRV as a marker for altered ANS responses and potential deficits in the linkage between their perceptions and actions.

However, some points remain to be elucidated, such as the cardiac autonomic control adjustment to the action of standing up, which is a simple maneuver performed several times a day by children and requires fast and compensatory autonomic adjustments to maintain homeostasis during gravitational changes in the human cardiovascular system (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). In addition, it is of interest to clarify whether the level of motor impairment (i.e., children with DCD vs. children at risk for DCD) is related to the severity of autonomic dysfunction. Moreover, heart rate (HR) modulation presents a nonlinear dynamic, which it is difficult to describe completely by linear methods (Signorini et al., 2001). Therefore, HRV nonlinear analyses have been applied and shown to provide complementary information about the underlying HR regulation mechanisms and to predict a pathological situation and/or a global depression of the organism (Goldberger, 1996; Guzzetti et al., 2005; Porta et al., 2009; Zamunér et al., 2015).

Elucidating whether children with DCD and at risk for DCD present abnormalities regarding cardiac autonomic control, may highlight the importance of a therapeutic approach aimed at improving cardiovascular health in this population. Therefore, this study sought to assess the cardiac autonomic control, by linear and nonlinear analysis, at supine and during an orthostatic stimulus in DCD, r-DCD and typically developed children. Our hypothesis is that children with DCD will present higher sympathetic, lower parasympathetic and lower complexity in the cardiac autonomic control in the supine position, as well as a blunted cardiac autonomic response to the orthostatic stimulus compared to typically developed children. Moreover, nonlinear analysis will provide complementary information to the linear analysis on cardiac autonomic control.

MATERIALS AND METHODS

Design and Population

This was a cross-sectional, case-control study. All children were recruited from elementary schools in São Carlos, São Paulo, Brazil. Ninety-seven children were screened for eligibility according to the guidelines for DCD diagnostic based on the Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-V) (American Psychiatric Association, 2013). According to the DSM-V, 25 children were classified as DCD, 32 children were classified as at risk for DCD (r-DCD) and 40 were classified as typically developed who were invited to take part in this study as controls. Out of this total, the parents of 47 children declined to participate in the study due the time mismatch. In the end, the final sample consisted of 13 DCD children (11 boys and 2 girls, aged 8.08 ± 0.79 years), 19 r-DCD children (13 boys and 6 girls, aged 8.10 ± 0.96 years) and 18 typically developed children that comprised the control group (CG, 10 boys, and 8 girls, aged 8.50 ± 0.96 years).

Children were included in the study if they satisfied the diagnostic criteria for DCD or r-DCD, based on DSM-V (American Psychiatric Association, 2013) and those with typical development were included in the CG. Exclusion criteria comprised a history of cardiovascular, respiratory, musculoskeletal, metabolic or neurological disorders, and continuous use of any medication.

All participants and their parents or guardians were informed as to the relevance of the research and about the experimental procedures. This study was carried out in accordance with the guidelines laid down in the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Federal University

of Sao Carlos (number 47091115.0.0000.5504). All parents gave written informed consent with a verbal assent from the children.

Instruments and Procedures

The anthropometric profile of the children included body weight (kg), height (cm) and waist circumference (cm) assessments. Body weight was measured using an electronic scale (Type Welmy W110H; range 0.01–200 kg; precision 0.01 kg) linked to a stadiometer (Type Welmy W110H; range 60–200 cm; precision 1 mm), which was used to measure the height. Waist circumference (WC) was determined using a measuring tape (Wiso; range 0–200 cm; precision 1 mm). Body mass index (BMI) was calculated as body weight/height squared (kg/m^2).

The children's motor performance concerning DCD identification was evaluated by the Movement Assessment Battery for Children–Second Edition (MABC-2). The MABC-2 is a standard instrument consisting of a set of eight motor tasks based on three motor domains: Manual Dexterity, Aiming & Catching and Balance to identify motor delay in children. According to the MABC-2 total scores, children were classified as: $\leq$56 points: children with significant movement delay (DCD children); from 57 to 67 points: children at risk of having movement delay (risk at DCD); and score above 67 points: children with no movement delay (typically developed children) (Henderson et al., 2007).

The children's general levels of physical activity were assessed using the Brazilian version of the Physical Activity Questionnaire for Children (PAQ-C) (Guedes and Guedes, 2015), which is a seven-day recall instrument. Responses were given on a five-point Likert scale. Each questionnaire item is scored between 1 (low) and 5 (high physical activity), and a mean score of all items constitutes the overall PAQ-C score. Higher values indicate better physical activity behavior (Kowalski et al., 1997). The PAQ-C was self-administered by the children's parents as secondary informants in a quiet room, since children under 10 years old often present difficulties remembering their previous daily activities (Silva and Malina, 2000). PAQ-C showed internal consistency values between 0.79 and 0.89 and test-retest reliability between 0.75 and 0.82. PAQ-C was previously validated using correlation analysis with the Godin and Shephard physical activity questionnaire ($r = 0.41$) and the Caltrac accelerometer ($r = 0.39$; Crocker et al., 1997).

Experimental Procedures

All experiments were carried out in the afternoon (13 p.m. to 18 p.m.) in order to minimize circadian changes. Room temperature was maintained at 22°C and relative air humidity at between 40 and 60%.

One week and the day prior to the cardiac autonomic control assessment, the children and their parents or guardians received relevant instructions to ensure a safe and satisfactory performance. Instructions were given to avoid the consumption of stimulating beverages or foods (e.g., coffee, soda, energy drinks, chocolate, black or green tea etc.,) and to suspend any major physical activity at least 24 h before the testing, to have a light meal before the testing and to have a good night's rest. All children were familiarized with the experimental protocol during a pilot test conducted 1 week prior to the study procedures.

R-R Intervals (RRi) Recording

Children were subjected to the recordings of RRi in order to assess the cardiac autonomic control.

Upon arrival in the laboratory, the participants rested for about 20 min in supine posture for the HR and blood pressure to stabilize and to return to their baseline conditions. Then, RRi were recorded for 15 min in supine position and 10 min in orthostatic position (active standing) with spontaneous breathing. The participants' breaths per minute were recorded during the entire collection period by the evaluator, by visual inspection of thoracoabdominal movements. Participants who had a respiratory rate below 9 breaths per minute (0.15 Hz) would be excluded due to the fact that breathing influences the frequency bands of spectral analysis (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Participants were requested not to talk or move in order to avoid alterations and artifacts in the RRi.

RRi data were collected at a sampling rate of 1,000 Hz, using a validated HR monitor and a transmitter belt (Polar V800, Polar Electro Co. Ltda. Kempele, Finland) (Giles et al., 2016) placed in the thoracic region at the fifth intercostal space.

HRV Analyses

HRV was analyzed by linear and nonlinear methods using software developed by Dr. Alberto Porta from University of Milan (Montano et al., 1994; Porta et al., 2007). A region of 256 consecutive beats with the greatest stability in the RR time series was found for all children and in all conditions (i.e., supine and orthostatic postures), and was selected for HRV analyses.

Linear Analysis

Spectral analysis was carried out by applying an autoregressive model in the previously selected RR section. The spectral components were obtained in low frequency (LF, 0.04–0.15 Hz) and high frequency bands (HF, 0.15–0.4 Hz) in absolute units (ms^2). Normalized units were computed by dividing the absolute potency of LF or HF components by the total variance of RRi (σ^2_{RR}) minus the very low frequency component (0.003–0.04 Hz) and multiplying this ratio by 100.

Nonlinear Analysis

The nonlinear methods used in the present study comprised symbolic analysis and Shannon entropy, both described in detail elsewhere (Porta et al., 2001).

Briefly, symbolic analysis comprises quantization of the RR time series selected for analysis in six uniformly distributed levels, where each beat receives a symbol (from 0 to 5). After that, four patterns are identified considering the sequences of three consecutive symbols: patterns without variation (0V), patterns with one variation (1V), patterns with two like variations (2LV), and patterns with two unlike variations (2UV). The percentage of each family's appearance is quantified. Previous studies (Guzzetti et al., 2001, 2005; Porta et al., 2001) have reported that the 0V% index represents sympathetic cardiac autonomic modulation, the

1V% represent both parasympathetic and sympathetic cardiac autonomic modulation, and the 2LV% 2UV% indices represent parasympathetic cardiac modulation.

Shannon entropy reflects the complexity of the RR time series by measuring the patterns' distribution complexity (sequences of three symbols). The presence of peaks (i.e., relevant patterns more frequently detected) or valleys (i.e., relevant missing or less frequent patterns) in the patterns' distribution determines the reduction of Shannon entropy. Conversely, maximal Shannon entropy is obtained when the patterns are identically distributed (Porta et al., 2001; Zamunér et al., 2015).

Statistical Analysis

Normality and homogeneity of variance assumptions were tested using the Shapiro-Wilk's and Levene's tests, respectively. Since several studies regarding DCD tend to group children with DCD and r-DCD, we performed a two-factor 2 × 2 mixed analysis of variance (ANOVA) with one between factor (CG vs. DCD and r-DCD grouped together) and one within factor (posture; supine vs. standing) to compare the differences between CG and DCD/r-DCD children in supine and orthostatic conditions. Following this, a two-factor 3 × 2 mixed ANOVA with one between factor (CG vs. DCD vs. r-DCD) and one within factor (posture; supine vs. standing) was performed to account for the severity of motor impairment. Where there was a significant interaction, analysis of the main effects was disregarded and the test for multiple comparisons with Bonferroni adjustment was performed. Assumptions for ANOVA were violated for σ^2_{RR}, LF and HF indices of HRV. Therefore, for these indices, between group comparisons and within group comparisons were performed using the Mann-Whitney and Wilcoxon tests, respectively, with Bonferroni adjustment *a priori*.

To control for a possible effect of confounding variables in the outcomes, a series of two-factor mixed analysis of covariance (ANCOVA) was computed considering gender, BMI, PAQ-C score and WC as covariates. Pearson's correlation coefficient (r) was used to assess the relationship between MABC-2 total scores and HRV indices. The significance level was set at 5%. Analyses were carried out using the SPSS (SPSS 22.0 version, Chicago, Illinois, USA).

RESULTS

Demographic Characteristics

Table 1 summarizes children's characterization by anthropometrical profile, motor performance assessed by MABC-2 total scores and levels of physical activity assessed by the PAQ-C scores in DCD, r-DCD, and CG. Significant difference between groups was only observed in MABC-2 total scores. As expected, CG presented higher MABC-2 total score compared to DCD ($p < 0.01$) and r-DCD ($p < 0.01$). Moreover, r-DCD presented higher MABC-2 total score compared to DCD ($p < 0.01$).

HRV Analysis

Results regarding interactions, main effects and multiple pairwise comparisons from the 2 × 2 ANOVA (i.e., considering DCD and r-DCD grouped together) are presented below and summarized in **Table 2**. **Table 3** summarizes only the multiple pairwise comparisons provided by the mixed model 3 × 2 ANOVA, while interactions and main effects results are discussed below.

Interaction Between Group (CG and DCD/r-DCD) and Posture

A significant group × posture interaction was observed for HR [$F_{(1, 48)} = 6.23$; $p = 0.02$], μRR [$F_{(1, 48)} = 8.03$; $p = 0.01$], LFnu [$F_{(1, 48)} = 7.05$; $p = 0.01$], HFnu [$F_{(1, 48)} = 7.05$; $p = 0.01$], Shannon entropy [$F_{(1, 50)} = 4.03$; $p = 0.049$], 0V [$F_{(1, 50)} = 5.17$; $p = 0.03$], 2LV [$F_{(1, 50)} = 5.26$; $p = 0.03$], and 2UV [$F_{(1, 50)} = 6.69$; $p = 0.01$].

Between group multiple pairwise comparisons (CG vs. DCD/r-DCD)

Planned pairwise comparisons revealed that at rest in supine posture, DCD/r-DCD presented lower Shannon entropy ($p = 0.01$), lower 2LV ($p = 0.004$), and higher 0V ($p = 0.01$) compared to the CG. No significant differences were found between CG and DCD/r-DCD in supine posture for HR ($p = 0.11$), μRR ($p = 0.09$), and linear indices of HRV (LFnu, $p = 0.18$ and HFnu, $p = 0.18$).

TABLE 1 | Demographic characteristics of children with typical development (CG), children at risk for developmental coordination disorder (r-DCD) and children with DCD.

Variables	CG ($n = 18$)	r-DCD ($n = 19$)	DCD ($n = 13$)	F value	p - value
Gender (M/F)	10/8	13/6	11/2	-	0.23*
MABC-2 total score	75.61 (6.63)#†	62.70 (3.22)†	46.91 (9.82)	69.62	<0.001
Weight (kg)	33.58 (9.08)	33.30 (10.38)	38.81 (14.62)	1.05	0.35
Height (cm)	136.97 (7.75)	133.97 (9.60)	134.41 (7.85)	0.61	0.54
BMI (kg/m^2)	17.74 (3.61)	18.22 (4.11)	21.01 (5.37)	2.27	0.11
WC (cm)	60.11 (10.17)	64.81 (11.73)	70.20 (12.10)	2.81	0.07
PAQ-C	2.91 (0.60)	2.46 (0.50)	2.46 (0.67)	2.69	0.07

*Data are presented as mean (standard deviation). Between group comparisons were performed by one-way ANOVA and Tukey post hoc test; *chi-square test. MABC-2, Movement Assessment Battery for Children – Second Edition; BMI, Body Mass Index; WC, Waist circumference; PAQ-C, Physical Activity Questionnaire for Children. #$p < 0.05$ vs. r-DCD; †$p < 0.05$ vs. DCD.*

TABLE 2 | Linear and nonlinear heart rate variability indices of children with typical development (CG), and children at risk for Developmental Coordination Disorder (r-DCD) and those with DCD grouped together.

	CG ($n = 18$)		DCD/r-DCD ($n = 32$)		*p*-value		
	Supine	**Standing**	**Supine**	**Standing**	**G**	**P**	**I**
HR (bpm)[a]	82 (11)	101 (14)*	89 (16)	99 (12)†	0.47	0.000	0.02
μRR (ms)[a]	750.1 (102.7)	601.3 (88.1)*	694.3 (107.13)	613.5 (73.4) †	0.38	0.000	0.01
σ^2RR (ms^2)[b,c]	2496.0 (1715.5–9370.5)	2005.7 (1303.8–4071.6)*	3342.5 (2084.1–6168.8)	1586.2 (985.4–3087.1) †	N/A	N/A	N/A
SPECTRAL ANALYSIS							
LF (ms^2)[b,c]	673.0 (455.5–2056)	524.5 (309.5–931.5)*	1086 (476.7–2872.7)	598 (383.5–1353)†	N/A	N/A	N/A
HF (ms^2)[b,c]	1572.5 (487.5–5265)	338.0 (164.5–509.7)*	1215.5 (707.7–3208)	397 (255.5–920.7)†	N/A	N/A	N/A
LF (nu)[a]	35.7 (15.6)	63.3 (15.7)*	42.7 (18.1)	58.3 (16.4) †	0.82	0.000	0.01
HF (nu)[a]	64.3 (15.6)	36.7 (15.7)*	57.3 (18.1)	41.7 (16.4)	0.82	0.000	0.01
NONLINEAR ANALYSES							
SE[a]	3.92 (0.36)#	3.46 (0.30)*	3.34 (0.8)	3.31 (0.62)	0.02	0.03	0.048
SYMBOLIC ANALYSIS							
0V (%)[a]	11.5 (8.4)#	29.6 (11.2)*	25.5 (20.8)	31.1 (15.4)	0.04	0.000	0.02
1V (%)[a]	44.6 (7.6)	47.1 (4.0)	43.2 (10.8)	45.2 (7.2)	0.36	0.18	0.87
2LV (%)[a]	19.3 (6.7)#	12.0 (5.1)*	12.5 (7.9)	10.2 (6.5)	0.01	0.000	0.01
2UV (%)[a]	24.6 (13.0)	11.2 (5.9)*	18.7 (10.4)	13.5 (7.7)†	0.44	0.000	0.01

*Values are expressed as mean (SD) or median (1st; 3rd quartile). P, posture main effect; G, group main effect; I, interaction; HR, heart rate; μRR, mean of RR intervals; σ^2RR, variance of RR intervals; LF, low frequency component of RR variability; HF, high frequency component of RR variability; nu, normalized units; SE, Shannon entropy; 0V, patterns with no variation; 1V, patterns with one variation; 2LV, patterns with two like variations; 2UV, patterns with two unlike variations; N/A, not applicable. *$p < 0.05$ CG supine vs. CG standing; †$p < 0.05$ DCD supine vs. DCD standing; #$p < 0.05$ CG supine vs. DCD supine.*

[a] *Two-factor mixed ANOVA with Bonferroni adjustment a posteriori.*

[b] *Mann-Whitney U test for between group comparisons;* [c] *Wilcoxon signed-rank test for within group comparisons.*

TABLE 3 | Linear and nonlinear heart rate variability indices from children with typical development (CG), children at risk for Developmental Coordination Disorder (r-DCD) and those with DCD.

	CG ($n = 18$)		r-DCD ($n = 13$)		DCD ($n = 19$)	
	Supine	**Standing**	**Supine**	**Standing**	**Supine**	**Standing**
HR (bpm)[a]	82 (11)	101 (14) †	86 (10)	100 (11) ‡	93 (22)	98 (12)
μRR (ms)[a]	750.1 (102.7)	601.3 (88.1)	706.7 (87.0)	607.6 (70.0)	676.2 (133.1)	622.1 (80.6)
σ^2RR (ms^2)[b,c]	2496.0 (1715.5–9370.5)	2005.7 (1303.8–4071.6) †	3154.7 (2349.6–6222.8)	1584.1 (952.8–3052.9) ‡	3530.4 (1860–6503.8)	1588.3 (1031.9–4211.8) §
LINEAR ANALYSIS						
LF (ms^2)[b,c]	673.0 (455.5–2056)	524.5 (309.5–931.5) ‡	1260 (539–2985)	563 (381–1377) ‡	727 (448–2849.5)	633.0 (381–1569) §
HF (ms^2)[b,c]	1572.5 (487.5–5265)	338.0 (164.5–509.7) †	1288 (768–3109)	312 (255–917) ‡	1150 (677–4118)	585.0 (274.5–1121.5) §
LF (nu)[a]	35.7 (15.6)	63.3 (15.7) †	42.1 (16.5)	57.9 (18.1)	43.6 (21.0)	58.8 (14.2) §
HF (nu)[a]	64.3 (15.6)	36.7 (15.7) †	57.9 (16.5)	42.1 (18.1)	56.3 (21.0)	41.2 (14.2) §
NONLINEAR ANALYSES						
SE[a]	3.92 (0.36)	3.46 (0.30)	3.30 (0.83)	3.35 (0.55)	3.34 (0.80)	3.25 (0.73)
SYMBOLIC ANALYSIS						
0V (%)[a]	11.5 (8.4)	29.6 (11.2)	24.5 (19.5)	31.0 (14.5)	26.9 (23.3)	31.2 (17.3)
1V (%)[a]	44.6 (7.6)	47.1 (4.0)	44.9 (10.4)	46.1 (6.5)	40.8 (11.2)	43.9 (8.2)
2LV (%)[a]	19.3 (6.7)*#	12.0 (5.1) †	12.7 (7.0)	9.8 (6.3)	12.3 (9.4)	10.7 (7.1)
2UV (%)[a]	24.6 (13.0)	11.2 (5.9) †	17.8 (8.0)	13.0 (5.4)	20.0 (13.5)	14.2 (10.3)

*Values are expressed as mean (SD) or median (1st; 3rd quartile). HR, heart rate; μRR, mean of RR intervals; σ^2RR, variance of RR intervals; LF, low frequency component of RR variability; HF, high frequency component of RR variability; nu, normalized units; SE, Shannon entropy; 0V, patterns with no variation; 1V, patterns with one variation; 2LV, patterns with two like variations; 2UV, patterns with two unlike variations. *$p < 0.05$ CG supine vs. r-DCD supine; #$p < 0.05$ CG supine vs. DCD supine; †$p < 0.05$ CG supine vs. CG standing; ‡$p < 0.05$ r-DCD supine vs. r-DCD standing. §$p < 0.05$ DCD supine vs. DCD standing.*

[a] *Two-factor mixed ANOVA with Bonferroni adjustment a posteriori.*

[b] *Mann-Whitney U test for between group comparisons.*

[c] *Wilcoxon signed-rank test for within group comparisons.*

Mann-Whitney test with a Bonferroni adjustment a priori revealed no significant differences between groups for σ^2RR ($p = 0.53$), LF ($p = 0.44$), and HF ($p = 0.76$).

Within group multiple pairwise comparisons (Supine vs. Standing)

Regarding the comparisons between postures, both groups (CG and DCD/r-DCD) significantly ($p < 0.05$) decreased μRR and HFnu, and increased LFnu when moving from supine to standing. However, the CG also decreased Shannon entropy ($p = 0.02$) and 2LV ($p < 0.0001$) and increased 0V ($p < 0.0001$) indices, which was not observed in the DCD/r-DCD group (Shannon entropy, $p = 0.75$; 2LV, $p = 0.10$, and 0V, $p = 0.10$).

Wilcoxon test with a Bonferroni adjustment a priori showed that both groups decreased σ^2RR, LF and HF during standing compared to the supine posture ($p < 0.01$).

Interaction Between Group (CG, r-DCD, and DCD) and Posture

Table 3 summarizes the results accounting for the severity of motor impairment (i.e., DCD and r-DCD group stratified). There was a significant group × posture interaction for HR [$F_{(1, 47)} = 4.30$; $p = 0.02$], μRR [$F_{(1, 47)} = 4.73$; $p = 0.01$], LFnu [$F_{(1, 47)} = 3.46$; $p = 0.04$], HFnu [$F_{(1, 47)} = 3.46$; $p = 0.04$], 2LV [$F_{(1, 47)} = 3.23$; $p = 0.048$], and 2UV [$F_{(1, 47)} = 3.23$; $p = 0.048$].

Regarding Shannon entropy and 0V, no significant group × posture interaction [$F_{(1, 47)} = 1.68$; $p = 0.20$; $F_{(1, 47)} = 2.42$; $p = 0.10$; respectively] or main effect of group [$F_{(2, 47)} = 3.07$; $p = 0.06$; $F_{(2, 47)} = 2.16$; $p = 0.13$; respectively] was found. A significant main effect of posture was observed for the 0V index [$F_{(1, 47)} = 11.91$; $p = 0.001$]. Therefore, regardless of group, a significant increase in 0V pattern, reflecting cardiac sympathetic modulation, was observed when moving from supine to standing.

Between group multiple pairwise comparisons (CG vs. r-DCD vs. DCD)

Pairwise comparisons revealed no significant differences ($p > 0.05$) between groups in supine or standing postures for HR, μRR and linear indices of HRV (LFnu, HFnu). Regarding nonlinear analysis, CG presented higher 2LV compared to r-DCD ($p = 0.03$) and DCD ($p = 0.046$) groups in supine position, reflecting higher parasympathetic modulation in CG.

Mann-Whitney test with a Bonferroni correction a priori revealed no significant differences between groups for σ^2RR, LF, and HF ($p > 0.05$).

Within group multiple pairwise comparisons (Supine vs. Standing)

Within group comparisons revealed significant increase in HR from supine to standing posture in CG ($p < 0.001$) and in the r-DCD group ($p < 0.001$) but not in the DCD group ($p = 0.17$). Symbolic analysis revealed a significant decrease in 2LV ($p < 0.0001$) and 2UV ($p < 0.0001$) from supine to standing in the CG, but not for the r-DCD (2LV, $p = 0.11$ and 2UV, $p = 0.06$) and DCD (2LV, $p = 0.44$ and 2UV, $p = 0.06$) groups.

All groups decreased σ^2RR, LF, HF, HFnu, and increased LFnu during standing compared to the supine posture ($p < 0.01$).

Confounding Factors

All results remained unchanged after performing an ANCOVA controlling for gender, BMI, PAQ-C score, and WC. Therefore, these results were not presented.

Relationship Between Motor Performance and Cardiac Autonomic Control

Correlation analysis revealed a significant association between MABC-2 total score and Shannon entropy ($r = 0.38$; $p < 0.01$; **Figure 1A**), 0V ($r = -0.36$; $p = 0.01$; **Figure 1B**) and 2LV ($r = 0.38$; $p < 0.01$; **Figure 1C**) assessed at supine posture.

DISCUSSION

The main findings of the present study were that: (1) DCD and r-DCD children grouped together presented higher sympathetic, lower parasympathetic, lower complexity of cardiac autonomic control in supine position and blunted autonomic adjustment to the orthostatic stimulus compared to typically developed children; (2) when stratified, r-DCD and especially the DCD group, presented blunted cardiac autonomic adjustment to the orthostatic stimulus, which was not observed in the CG group, reflecting that the severity of motor impairment might be related to the severity of autonomic dysfunction; (3) significant relationships between MABC-2 total score and nonlinear indices of HRV were observed, suggesting that the lower the motor performance the lower the complexity of the cardiac autonomic control (Shannon entropy) and the cardiac parasympathetic modulation (2LV) and the higher the cardiac sympathetic modulation (0V); and (4) nonlinear analysis of HRV provided non-redundant and complementary information about cardiac autonomic control, depicting cardiac autonomic abnormalities not identified by traditional linear methods, even in children with less severe motor impairment (i.e., r-DCD).

To the best of our knowledge, this is the first study addressing cardiac autonomic control by linear and nonlinear analysis of HRV in children with DCD and r-DCD. However, previous studies have addressed cardiovascular variables in this population (Chirico et al., 2012; Coverdale et al., 2012; Chen et al., 2015). Coverdale et al. (2012) studied the cardiac autonomic control and baroreflex sensitivity in the resting supine condition in adolescents with suspect and probable DCD. Despite being a different population, our results partially corroborate their findings. The authors (Coverdale et al., 2012) reported no significant differences in cardiac autonomic control, quantified by linear HRV indices, between controls and suspect and/or probable DCD groups in supine position. On the other hand, baroreflex sensitivity was lower in the probable DCD group compared to the CG and suspect DCD, which was mainly attributed to the higher body fat percentage in this group. Chirico et al. (2011) aimed to compare the heart left ventricular structure and function between children with DCD and healthy controls. The authors reported significantly elevated end-diastolic volume, diastolic chamber size, stroke volume, and cardiac output in

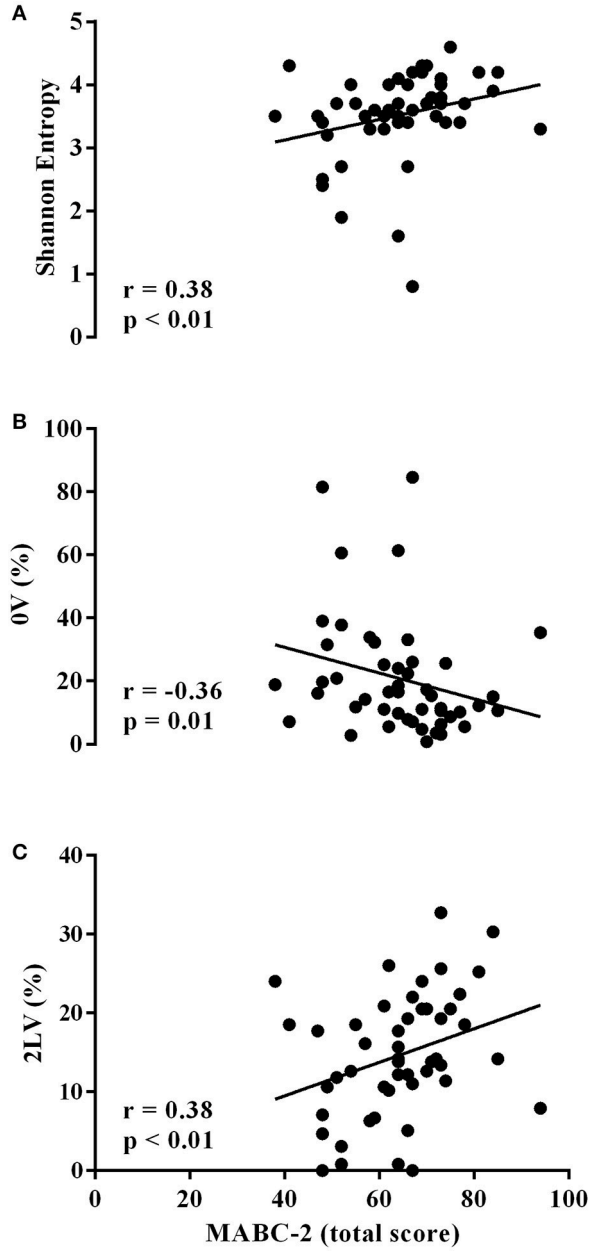

FIGURE 1 | Scatter plots of Shannon entropy **(A)**, 0V pattern **(B)** and 2LV pattern of symbolic analysis **(C)** at rest on MABC-2 total score are shown. Each solid circle is relevant to a pair (MABC-2, nonlinear heart rate variability indices) computed over a single individual.

children with probable DCD, suggesting obesity related changes in the left ventricle. Moreover, Chirico et al. (2012) found that elevated fat mass in adolescents with probable DCD contributes to higher cardiac output and left ventricle mass over time compared to typically developed controls. Nevertheless, in the present study the CG was matched to the DCD group for BMI. Therefore, anthropometric characteristics, such as BMI, weight and WC, were not different between groups. Thus, obesity may not be the only factor explaining the current results.

Another interesting study in this field was carried out by Chen et al. (2015). The authors aimed to study cardiac autonomic control in children with or at risk for DCD during cognitive tasks at different levels of difficulty. The authors found higher cardiac sympathetic modulation in children with DCD in comparison to typically developed children. In addition, the authors reported a blunted cardiac autonomic adjustment to some cognitive tasks in the DCD group compared to controls. These results are in agreement with our findings, since a blunted cardiac autonomic response, characterized by limited decrease in parasympathetic, as observed by the indices 2LV and 2UV, and limited increase in sympathetic cardiac modulation, as observed by the 0V index, were also observed in DCD and r-DCD groups during gravitational stimulus. The authors suggested that the higher cardiac sympathetic modulation in DCD children might be due to lower levels of aerobic fitness. In the present study, although not statistically significant, r-DCD and DCD groups presented lower levels of physical activity, as assessed by the PAQ-C. However, the results remained unchanged after considering the level of physical activity as covariate. Therefore, some hypothesis other than obesity and aerobic fitness underlying the present results should also be considered.

One possible explanation might be related to the patterns of connectivity and neural recruitment observed in children with DCD. Wilson et al. (2017) revised systematic neuroimaging data from studies performed with DCD and stated that the neural activity of these children is similar to that observed in children with mild cerebral palsy and born preterm. Indeed, several studies have reported cardiac autonomic abnormalities in those born preterm (Clairambault et al., 1992; van Ravenswaaij-Arts et al., 1995) and in children with cerebral palsy (Park et al., 2002; Zamunér et al., 2011; Amichai and Katz-Leurer, 2014), characterized by greater sympathetic, lower parasympathetic and lower complexity in cardiac autonomic modulation, compared to typically developed children. In addition, impaired autonomic adjustment to the postural changes was also observed in children with cerebral palsy (Park et al., 2002; Zamunér et al., 2011), suggesting that sympathetic activation was not enough to overcome the orthostatic stress imposed on these children. These findings corroborate our results.

Moreover, Zamunér et al. (2011) reported that the more severe the child's motor impairment, the lower the HRV, which was also found in the present study, since cardiac autonomic dysfunction was more pronounced in the DCD group than in the r-DCD group. In addition, significant relationships between motor performance and nonlinear HRV indices were also observed. Regarding cerebral palsy, the authors justified their results suggesting a possible effect from the loss of hemispherical influences on autonomic control resulting from existing cerebral lesions. Indeed, some authors suggest that in children with DCD the neural substrate mimics that of cerebral palsy (Peters et al., 2013) with lower brain activity in some cortical areas (Querne et al., 2008; Kashiwagi et al., 2009) responsible for body adjustment functions, which also could account for abnormalities in cardiac autonomic control and its relationship with the severity of motor impairment in these children. Therefore, future

studies should address whether there is a relationship between neuroimaging data, neural activity and autonomic dysfunction in DCD children.

Another noteworthy result was that nonlinear analysis of HRV provided relevant complementary information about cardiac autonomic regulation, identifying cardiac autonomic abnormalities not detected by traditional linear methods, even in children with less severe motor impairment (i.e., r-DCD). Nonlinear methods have been shown to better describe nonlinear dynamics in RRi time series than linear methods (Voss et al., 1996; Zamunér et al., 2013, 2015), thus providing additional insights regarding cardiac autonomic regulation. Shannon entropy's analysis revealed that r-DCD/DCD children presented reduced complexity in cardiac autonomic regulation. Several studies have reported that a decrease in complexity indices might represent a depressed organ function, a loss of interaction between subsystems, an overwhelming action of a subsystem over others and an impairment of regulatory mechanisms, therefore constituting a clear hallmark of a pathological condition (Porta et al., 2009; Zamunér et al., 2013, 2015). Furthermore, symbolic analysis enabled the quantification of nonreciprocal changes in sympathetic (0V pattern) and parasympathetic (2LV and 2UV patterns) cardiac autonomic control, differently from spectral analysis, which provides more information regarding the parasympathetic branch (Porta et al., 2009).

Despite these interesting results, some study limitations need to be acknowledged. It is worth mentioning that we observed significant group × posture interaction for HR, revealing a blunted increase of HR to the orthostatic stimulus in the DCD group. It is well known that linear and some nonlinear indices of HRV are significantly correlated with average HR (Sacha and Pluta, 2008; Sacha et al., 2013; Bolea et al., 2016), including in pediatric population (Gasior et al., 2015). Therefore, several mathematical procedures have been proposed in order to attenuate the HRV dependence on HR (Sacha et al., 2013; Monfredi et al., 2014; Bolea et al., 2016). Nevertheless, to the best of our knowledge, no procedures have been proposed to normalize the nonlinear indices used in the present study (i.e. Shannon entropy and symbolic analysis indices). Thus, HRV normalization was not performed. However, a possible influence of HR should not be a bias on the present study due to no significant differences between groups regarding HR. Moreover, HRV indices, specially the nonlinear ones, provided additional information to the HR itself. Even though, it is important to highlight that future studies should quantify these nonlinear indices dependence on HR and propose methods to address this issue.

Another limitation of the present study was that from the 97 children screened for eligibility, 48% of the parents declined to participate in the study, which may have led to a possible sample selection bias. Even though, this is an important outcome drawing attention to the need to improve parents' awareness and education about DCD. Moreover, the difference between groups regarding gender should be mentioned. Although it was not significant and results remained unchanged after considering it as covariate, future studies should address this issue. Furthermore, 24-h HRV should also be considered in future studies in order to elucidate possible cardiac autonomic control abnormalities in this population during daily life and sleep.

In conclusion, r-DCD and especially DCD children presented higher sympathetic, lower parasympathetic, lower complexity of the cardiac autonomic control in supine position and blunted autonomic adjustment to the orthostatic stimulus compared to the typically developed children. In addition, the lower the motor performance, the lower the complexity of the cardiac autonomic control and the cardiac parasympathetic modulation; and the higher the cardiac sympathetic control. Thus, since the assessment of cardiac autonomic control is easily performed by noninvasive method and provides an important parameter for cardiovascular risk prognosis, it should be part of routine assessments in this population.

AUTHOR CONTRIBUTIONS

JC, AZ, ES, and ET designed the study. JC and AZ performed the experiments and drafted the manuscript. JC, AZ, and BM analyzed the data. JC, AZ, BM, ES, and ET interpreted the data. JC, AZ, BM, ES, and ET revised the manuscript. JC, AZ, BM, ES, and ET approved the final version of the manuscript to be published.

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REVIEW
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A Meta-Analysis on Sex Differences in Resting-State Vagal Activity in Children and Adolescents

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Lower vagal activity is associated with psychopathology independent of age. Research suggests that alterations of vagal activity precede the development of psychopathology. The present review aimed to quantify sex differences in vagal activity in children and adolescents. Studies reporting on sex differences on measures of vagally-mediated heart rate variability derived from short-term recordings under resting conditions in boys and girls were included. Drawing on data from more than 5,000 children and adolescents, we provide evidence that healthy young girls display lower vagal activity and greater mean heart rate compared to boys, a finding that may have implications for risk associated with the development of internalizing psychopathology and somatic ill-health.

Keywords: vagal activity, heart rate variability, sex differences, children, adolescents

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INTRODUCTION

There are marked sex differences in the prevalence of adolescent psychiatric disorders. For example, mood, anxiety, and eating disorders usually have an onset during adolescence, and are more prevalent among girls (Zahn-Waxler et al., 2008; Merikangas et al., 2009).

Reduced resting state vagal activity, indexed by measures of high frequency heart rate variability [HF-HRV; rapid variability in heart rate (HR) observed with spontaneous respiration that is also referred to as respiratory sinus arrhythmia; RSA], has been linked to a variety of mental health conditions in adults (Malik and Camm, 2007). For example, reduced HF-HRV compared to healthy controls has been reported in depression (Kemp et al., 2010), anxiety disorders (Chalmers et al., 2014), and Borderline Personality Disorder (BPD) (Koenig et al., 2016b). Among children and adolescents, lower resting HF-HRV is observed in autism spectrum disorder (Neuhaus et al., 2014), conduct disorder (Beauchaine et al., 2001, 2007), BPD (Koenig et al., 2017b), and depression (Koenig et al., 2016a) but not attention deficit hyperactivity disorder (Koenig et al., 2017a). A study of autonomic function in a population cohort reported that HF-HRV was higher in adolescents with externalizing problems relative to adolescents with internalizing problems (Dietrich et al., 2007). Resting state vagal activity, indexed by HF-HRV, has been shown to be related to individual differences in the perception of emotional stimuli (Park et al., 2013; Park and Thayer, 2014) and to predict affective instability in daily life (Koval et al., 2013). As several studies have shown, reduced HF-HRV is associated with difficulties in emotion regulation among child, adolescent, and adult samples (see e.g., Berna et al., 2014; Beauchaine, 2015; Williams et al., 2015). Thus, HF-HRV may have a particular association to the internalizing disorders associated with difficulties in emotion

regulation. With respect to temporal sequencing, research from the Whitehall II longitudinal study suggests that altered vagal activity, as indexed by HF-HRV, may precede the development of internalizing psychopathology such as depression (Jandackova et al., 2016). Similar, it has recently been shown, that reduced HF-HRV significantly predicted increased depressive symptoms across 1 year in a sample of 73 adolescents (Vazquez et al., 2016). Beyond psychiatric disorders, decreased resting state HRV is associated with a poorly functioning anti-inflammatory reflex (Pavlov and Tracey, 2012), increasing risk for physical ill-health in general (see Kemp and Quintana, 2013 for a review) and cardiovascular disease (CVD) in particular, the latter being the leading cause of death and disability worldwide (Thayer et al., 2010).

Given that parasympathetic and sympathetic influence on the heart operate by different signaling transmitters (i.e., acetylcholine and norepinephrine, respectively) with different latencies of onset, only parasympathetic influences can account for rapid changes in HR and its variability that are often observed during spontaneous respiration (Berntson et al., 1997). As such, power in the HF-HRV band and time-domain measures reflecting fast changes in the inter-beat interval (IBI) series (i.e., the root mean square of successive differences, RMSSD) are regarded as indices of vagal activity under resting conditions. In support of this, HF-HRV is nearly abolished by cholinergic blockade and functional vagotomy, confirming that HF-HRV is mediated primarily by change in vagal cardiac nerve traffic (Pomeranz et al., 1985; Berntson et al., 1997). While the interpretation of activity in the low-frequency (LF) band remains equivocal (Goldstein et al., 2011; Rahman et al., 2011), it is often considered to reflect both, activity of the sympathetic and parasympathetic branches of the autonomic nervous system (ANS).

Recently, we demonstrated substantial sex differences in time- and frequency-domain measures of HRV in healthy human adults using a meta-analytical approach drawing on data from a cumulative total of 296,247 subjects (Koenig and Thayer, 2016). Relative to men, the autonomic control of the women's heart is characterized by significantly less total power in the power spectral density (PSD) containing greater HF- and less LF-power. This was further reflected in a lower LF/HF ratio. Despite greater mean HR, women show higher levels of vagal parasympathetic activity relative to men. Most interestingly, sex differences emerged as a function of age and were more pronounced in older samples compared to younger samples. These results have important implications for research into the development of psychiatric disorders. Given that the prevalence of mood and anxiety disorders is higher in girls while the prevalence of externalizing disorders and substance abuse is higher in boys (Seedat et al., 2009), one would expect girls to show relatively lower parasympathetic vagal activity. Contributing to the *Frontiers Research Topic* addressing "*Heart Rate Variability and other Autonomic Markers in Children and Adolescents,*" the aim of the present review and meta-analysis was therefore to rereview the existing evidence on sex-differences in vagal activity, indexed by measures of vagally-mediated HRV, in children and adolescents under the age of 18. Sex differences in vagal activity at such young age may provide one mechanism contributing to differences in the prevalence of psychiatric disorders in children and adolescents.

METHODS

Systematic Search of the Literature

The database of studies of a previous meta-analysis (Koenig and Thayer, 2016) was used for the present reanalysis. The initial systematic search of the literature was based on a review of four digital databases [PubMed, Web of Science (WOS), PsycINFO, and CINAHL Plus]. As indicated in the original report, the number of initial hits was recorded and after removing duplicates, abstracts of all identified articles were screened based on pre-defined inclusion criteria (Koenig and Thayer, 2016). Details on the literature search and criteria for inclusion are published elsewhere (Koenig and Thayer, 2016). For the present analysis, studies from the existing database were selected if the full-text reported (i) sufficient descriptive data (*see below*) of (ii) any given measure of vagally-mediated HRV, (iii) separately for boys and girls (sample mean age <18 years). Studies reporting overlapping samples were excluded. In case overlapping samples were reported by multiple titles, the earliest published report was included.

Data Extraction and Meta-Analysis

The name of the authors, the year of publication, sample size (total and by sex), and age were retrieved from all included studies. For the present meta-analysis we extracted all measures of vagally-mediated HRV reported, including time- (i.e., RMSSD) and frequency-domain (i.e., HF-HRV, RSA) measures. If studies reported measures of vagally-mediated HRV we also extracted data on mean HR and the mean IBI if reported. Descriptive data on the reported measures were extracted for female and male subjects separately. Measures of short-term recordings only were extracted. It is noted that guidelines for the measurement of HRV suggest "*analyses of short- and long-term electrocardiograms should always be strictly distinguished*" (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology, 1996). For the present meta-analysis we focus on short-term recordings of resting state HF-HRV, as these are sought to reflect important trait influence (Bertsch et al., 2012) and are most frequently reported in the psychiatric literature.

Meta-analytical effect size estimates were based on means, standard deviations (*SD*), and the sample size (*n*). In case descriptive data was available other than as mean and SDs, data transformations were applied (Hozo et al., 2005; Wiebe et al., 2006; Higgins and Green, 2011). True effect estimates were computed as adjusted standardized mean differences (SMD, Hedge's g) for all measures. We undertook meta-analyses using *random-effect* models. Heterogeneity or inconsistency among trials in the magnitude or direction of effects estimated was investigated. Heterogeneity was assessed using the standard I^2 *index*, Chi^2, and Tau^2 tests (Higgins and Thompson, 2002). Bias was further examined using funnel plots, illustrating the

effect size (SMD) against standard error for asymmetry. Meta-analytic computations were performed using RevMan (*Version 5.3.4, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014*).

RESULTS

Included Studies

In historical order, Hedelin et al. (2000) assessed the effect of competitive cross-country skiing on HRV by testing HRV at rest and in response to tilt and exercise challenges before and after ski seasons among 17 adolescents. Grandjean et al. (2004) evaluated whether heart function in childhood is affected by exposure to methylmercury from seafood using a prospective cohort of 878 children assessed at 7 and 14 years of age. Cardiovascular function was assessed at both time points while children were in a relaxed supine condition. Gutin et al. (2005) assessed the effect of race, sex, physical activity, cardiovascular fitness, and adiposity on cardiac autonomic modulation in 304 healthy adolescents between 14 and 18 years of age. Wang et al. (2005) assessed whether HRV differed in Black and White children. Brunetto et al. (2005) evaluated the effects of aerobic fitness on HF-HRV in a sample of 41 adolescents aged 12–17 years. Participants were separated into tertiles based on aerobic fitness and HF-HRV was assessed at rest and in response to a maximal treadmill exercise test. The study by Greaves-Lord et al. (2007) evaluated the association between hyperarousal and symptoms of anxious and depressed mood using a nationally representative prospective cohort of Dutch children between 10 and 13 years of age. Reed et al. (2006) tested whether HRV in children differed by ethnicity (Caucasian and Asian) and sex. Sharshenova et al. (2006) recruited children between 9 and 10 years of age from three countries of different altitudes (1,650, 1,740, 2,030 m) to evaluate the effects of sex and altitude on HF-HRV.

Moodithaya and Avadhany (2012) evaluated sex differences in age-associated change in cardiac autonomic activity assessed via HRV using a cross sectional design. Michels et al. (2013) sought to provide age and sex specific reference values for short-term HRV data in children between 5 and 10 years of age. A secondary goal was to evaluate associations between HRV, physical fitness, BMI, and body composition. Tsao et al. (2013) assessed the effect of sex and age on conditioned pain modulation in a sample of 133 healthy children. Koch and Pollatos (2014) assessed the association between cardiac sensitivity and emotional intelligence in a sample of 1,350 children between 6 and 11 years of age. Jarrin et al. (2015) sought to derive normative HRV values stratified by age, sex, and HR for a population-based sample of children in Quebec. A summary of all included studies is provided in **Table 1**.

Excluded Studies

Of the studies conducted in children/adolescents, five were excluded due to reporting on long-term recordings of HRV only (Han et al., 2000; Silvetti et al., 2001; Faulkner et al., 2003; Aziz et al., 2012; Rodríguez-Colón et al., 2014). Four studies had to be excluded for not reporting a measure of vagally-mediated HRV (Pikkujämsä et al., 1999; Lauritzen et al., 2008; Eyre et al., 2013; Faust et al., 2013). Further, one study reporting on fetuses was excluded (Kwon et al., 2014).

Sex Differences in Vagal Activity

Of studies screened for eligibility, 13 (Hedelin et al., 2000; Grandjean et al., 2004; Brunetto et al., 2005; Gutin et al., 2005; Wang et al., 2005; Reed et al., 2006; Sharshenova et al., 2006; Greaves-Lord et al., 2007; Moodithaya and Avadhany, 2012; Michels et al., 2013; Tsao et al., 2013; Koch and Pollatos, 2014; Jarrin et al., 2015) reported measures of vagally-mediated HRV derived from short-term recordings, yielding a total of 11 pooled comparisons for HF-HRV (**Figure 1**) and 8 comparisons for RMSSD (**Figure 2**). Meta-analysis showed a significant main effect for HF-HRV ($Z = 2.11$, $p = 0.03$; Hedges' $g = -0.10$, 95%CI [−0.20;−0.01], $k = 11$), comparing girls ($n = 3{,}004$) to boys ($n = 2{,}802$). Meta-analysis on RMSSD also yielded a significant main effect ($Z = 6.53$, $p < 0.0001$; Hedges' $g = -0.21$, 95%CI [−0.28;−0.15], $k = 8$), indicating lower resting state vagal activity in girls ($n = 1{,}952$) compared to boys ($n = 1{,}810$). While heterogeneity was present in the analysis of HF-HRV (**Figure 1**), respective tests indicated no heterogeneity for the analysis of RMSSD (**Figure 2**). Visual inspection of Funnel-Plots, revealed no significant publication bias for RMSSD but one outlier (**Figure 1**) in the analysis of HF-HRV (Hedelin et al., 2000). After removal of the outlier from analysis, meta-analysis on HF-HRV still showed a significant main effect ($Z = 2.68$, $p = 0.007$; Hedges' $g = -0.11$, 95%CI [−0.20;−0.03], $k = 10$; girls = 2,993; boys $n = 2{,}794$). Heterogeneity was moderate after removal of the outlier $I^2 = 52\%$.

Sex Differences in Heart Rate and Mean Inter-Beat-Interval

Of the included studies, eight also reported data on mean HR (Grandjean et al., 2004; Gutin et al., 2005; Reed et al., 2006; Greaves-Lord et al., 2007; Moodithaya and Avadhany, 2012; Michels et al., 2013; Koch and Pollatos, 2014; Jarrin et al., 2015) and five (Brunetto et al., 2005; Wang et al., 2005; Sharshenova et al., 2006; Michels et al., 2013; Jarrin et al., 2015) on mean IBI. Meta-analysis showed a significant main effect for mean HR ($Z = 8.02$, $p < 0.0001$; Hedges' $g = 0.25$, 95%CI [0.19;0.31], $k = 8$), indicating lower mean HR in boys ($n = 2{,}567$) compared to girls ($n = 2{,}761$), as illustrated in **Figure 3**. Meta-analysis on mean IBI also yielded a significant main effect ($Z = 6.06$, $p < 0.0001$; Hedges' $g = -0.38$, 95%CI [−0.51;−0.26], $k = 5$), indicating lower mean IBI in girls ($n = 1{,}028$) compared to boys ($n = 968$; **Figure 4**). No significant heterogeneity or publication bias was present for analysis on mean HR (**Figure 3**) and mean IBI (**Figure 4**).

DISCUSSION

Main Findings

We observed a small to moderate effect of sex on cardiac vagal activity derived from short-term, time-, and frequency-domain measures of HF-HRV among children and adolescents, such that girls displayed lower resting state cardiac vagal activity and greater mean HR relative to boys. Seven of the included studies

TABLE 1 | Study and sample characteristics of studies reporting short-term heart rate variability; HF-HRV: high-frequency heart rate variability; RMSSD: root mean square of successive differences; NR: not reported; HR: heart rate; IBI: inter-beat-interval.

Author and Year	Country	Sample size (Female)	Age	Inclusions	Exclusions	Other important factors	Adjustment for potential confounds	HRV recording	Recording duration	Tasks performed	Recording equipment	Processing equipment	HR/HRV metric	Breathing	HF-HRV frequency band
Brunetto et al., 2005	Brasil	41 (21)	All: 15.3 (0.96);	Healthy	Smoker, Obesity, Hypertension, Medications, Diabetes, Syncope	Recruited from public schools.	No caffeine or alcohol on day of testing. No strenuous exercise on day prior to testing. Meal on day of testing was "light"	10-min resting baseline	5-min	Head tilt and incremental exercise task	Polar S810 (Polar Electro Oy, Finland)	Software developed by Yamamoto & Hughson	RMSSD; IBI	15 breaths per minute	0.15–0.4 Hz
Grandjean et al., 2004	Denmark	878 (440)	Sample assessed at 6.84 (0.31)– and 13.83 (0.32)–years	Sampled from a longitudinal cohort from the Faroe Islands	Neurological or other serious diseases; Low birth-weight.	NR	NR	Supine position	5-min	None	NEC-Sanei 1271SP ECG amplifier (Tokyo, Japan)	NR	HF-HRV; HR	Spontaneous respiration	0.15–0.4 Hz
Greaves-Lord et al., 2007	Netherlands	1027 (543)	All: 11(0.5)	10–13 years of age; Members of prospective "TRIALS" cohort	Severe mental retardation; Serious physical illness	NR	NR	Supine position	4-min	2-min standing	DAS-12 (Keithley Instruments Cleveland, OH)	CARSPAN Software	HF-HRV; HR	Spontaneous respiration	>0.14 Hz
Gutin et al., 2005	US	304 (171)	All: 16.24 (1.21);	Blacks and Whites; 14–18 years of age; Healthy	NR	NR	NR	Supine position with 10-min quiet rest	256 R-R intervals	None	Schiller electrocardiogrphic system (Schiller Ag, Baar, Switzerland)	NR	HF-HRV; RMSSD; HR	Spontaneous respiration	0.15–0.4 Hz
Jarrin et al., 2015	1036(555)	Canada	All: 10.2 (0.3);	Participants in a nationally representative Quebec longitudinal study	Medical pathology; born <24 or > 42 weeks gestation; Remote living	Representative random sampling was performed in Quebec Systems, Milwaukee, Wisconsin, USA)	Physical activity, medication, caffeine intake within 24-h of testing	listed as "standardized protocol" Systems, Milwaukee, Wisconsin, USA)	1-h	None	Marquette MARS 8500 Holter Monitor (Marquette Medical	Marquette Analysis workstation (Marquette Medical	HF-HRV; RMSSD; IBI; HR	NR	0.15–0.4 Hz
Hedelin et al., 2000	Sweden	17 (9)	NR	16–19 years of age; competitive cross-country skier	NR	Resting values were taken during controlled breathing	NR	Controlled breathing in supine position	5-min	Tilt table and exercise test	NR	Matlab (Mathworks Inc, Natick, MA)	HF-HRV; no HR/IBI (only during exercise)	12 breaths per minute	0.15–0.45 Hz
Koch and Pollatos, 2014	Germany	1350 (693)	All: 8.39 (0.94);	Members of longitudinal PIER study.	Missing data and technical problems (n = 305)	NR	NR	Resting	3-min	Heartbeat perception task	Polar RS800CX (Polar Electro Oy, Finland)	Polar ProTrainer 5	HF-HRV; RMSSD; HR	Spontaneous respiration	0.15–0.4 Hz
Michels et al., 2013	Belgium	460 (220)	All: 8.05 (NR)	Members of Belgin control region. 5-10 years of age;	Cardiovascular disease; Diabetes; Low quality HRV measurement	Physical activity; Normal breathing; Movement; Time of day	NR	Supine position	10-min	None	Polar Wearlink 31 (Polar Electro Oy, Finland)	Reported as "University of Kuopio Software"	HF-HRV; RMSSD; IBI; HR	Spontaneous respiration	0.15–0.4 Hz

(Continued)

TABLE 1 | Continued

Author and Year	Country	Sample size (Female)	Age	Inclusions	Exclusions	Other important factors	Adjustment for potential confounds	HRV recording	Recording duration	Tasks performed	Recording equipment	Processing equipment	HR/HRV metric	Breathing	HF-HRV frequency band
Moodithaya and Avadhany, 2012	India	60 (30)	All: 9.4 (0.3);	Students and faculty at an institute in Bangalore	Hypertension; Diabetes; Chronic Disease; Oral contraceptive	Tested in morning after fasting; Refrain from smoking, caffeine, strenuous exercise (23-h)		Supine position	5-min	None	Biopac MP30 (Bipac Systems Inc, Santa Barbara, CA).	NR	HF-HRV; HR	Spontaneous respiration	0.15–0.4 Hz
Reed et al., 2006	62 (32)	Canada	All: 10.35 (0.6);	Participants drawn for a larger cohort enrolled in a school-based exercise program	CVD	NR	Tested in morning at school; No caffeine 2-h prior	Supine position	6-min (5 used)	None	Polar S810 (Polar Electro Oy, Finland)	Biomedicals Signal Analysis	HF-HRV; RMSSD; HR	NR	0.15–0.5 Hz
Sharshenova et al., 2006	113 (58)	listed as "3 countries"	All: 9 to 10 years of age	Healthy; Free of Chronic Illness	NR	Permanent residents from three countries tested at three different altitudes.		At rest	NR	during standing	NR	Reported as "specialy developed software"	HF-HRV; IBI	NR	0.15–0.4 Hz
Tsao et al., 2013	133 (70)	US	All: 13.0 (2.9);	Healthy children between 8 and 17 years of age	Medications; Chronic pain; Acute Illness; Developmental Delay	Data taken from a study of laboratory pain responses in children.	NR	Seated quietly watching neutral video	5-min	Laboratory pain tasks	Biopac System (Bipac Systems Inc, Santa Barbara, CA).	Kubios HRV Software	RMSSD; NO HR/IBI	NR	
Wang et al., 2005	385 (196)	US	All: 16.0 (2.0)	Members of Georgia Longitudinal Study or the George Cardiovascular Twin Study	Healthy; Free of Chronic Illness	Results are reported for White and Black participants	NR	Supine poition	256 R-R intervals	None	Schiller electrocardiogrphic system (Schiller Ag, Baar, Switzerland)	NR	HF-HRV; RMSSD; IBI	NR	0.15–0.4 Hz

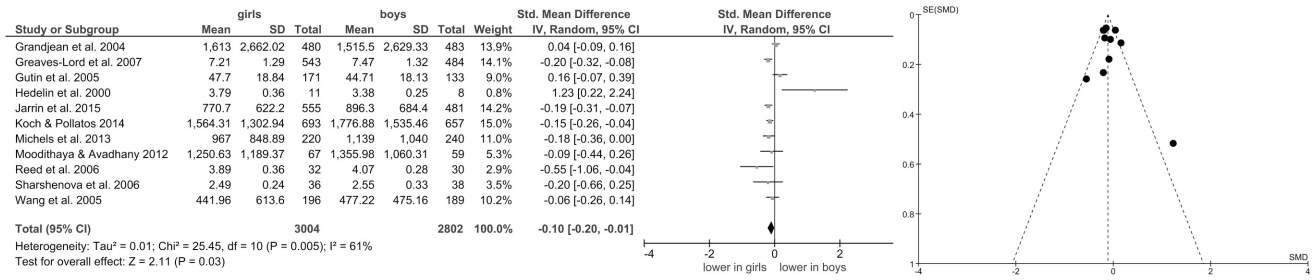

FIGURE 1 | Forrest- and Funnel-Plot from Meta-Analysis on Short-Term Resting State HF-HRV; Grandjean et al. (2004): HF in ms^2; data pooled for follow-up assessment (7 and 14 years of age), n of those with two assessments used before exclusion (unclear sex of excluded subjects); HF reported as geometric mean and interquartile range, transformed before pooling and analysis; Greaves-Lord et al. (2007): RSA in log ms^2; supine data used for analysis; Gutin et al. (2005): HF in normalized units; data pooled across ethnic groups; Hedelin et al. (2000): HF in log ms^2; supine data before training period (test 1) used; data reported as mean and range, transformed before analysis; Jarrin et al. (2015): HF in ms^2; Koch and Pollatos (2014): HF in ms^2; Michels et al. (2013): HF in ms^2; data reported as mean and interquartile range, transformed before analysis; Moodithaya and Avadhany (2012): HF in ms^2; data pooled for children and adolescents; data reported as mean and standard error of the mean, transformed before analysis; Reed et al. (2006): HF in log ms^2; data pooled across ethnic groups; Sharshenova et al. (2006): HF in log ms^2; supine data used for analysis; Wang et al. (2005): HF in ms^2; data pooled across ethnic groups.

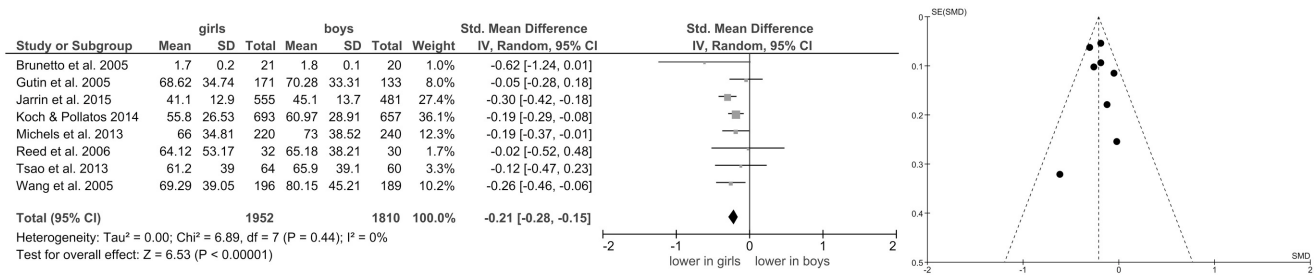

FIGURE 2 | Forrest- and Funnel-Plot from Meta-Analysis on Short-Term Resting State RMSSD; Brunetto et al. (2005): supine data used for analysis; Gutin et al. (2005): data pooled across ethnic groups; Jarrin et al. (2015): data used as reported; Koch and Pollatos (2014): data used as reported; Michels et al. (2013): data reported as mean and interquartile range, transformed before analysis; Reed et al. (2006): data pooled across ethnic groups; Tsao et al. (2013): pre-task baseline data used; Wang et al. (2005): data pooled across ethnic groups.

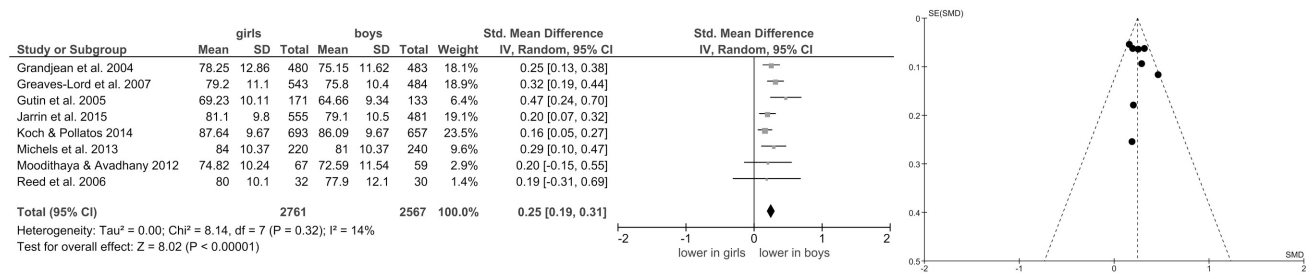

FIGURE 3 | Forrest- and Funnel-Plot from Meta-Analysis on Short-Term Resting State HR; Grandjean et al. (2004): data pooled for follow-up assessment (7 and 14 years of age), n of those with two assessments used before exclusion (unclear sex of excluded subjects); Greaves-Lord et al. (2007): supine data used for analysis; Gutin et al. (2005): data pooled across ethnic groups; Jarrin et al. (2015): data used as reported; Koch and Pollatos (2014): data used as reported; Michels et al. (2013): data reported as mean and interquartile range, transformed before analysis; Moodithaya and Avadhany (2012): data pooled for children and adolescents; data reported as mean and standard error of the mean, transformed before analysis; Reed et al. (2006): data pooled across ethnic groups.

initially reported significant sex differences on measures of vagal activity (Hedelin et al., 2000; Grandjean et al., 2004; Sharshenova et al., 2006; Moodithaya and Avadhany, 2012; Michels et al., 2013; Koch and Pollatos, 2014; Jarrin et al., 2015), with all but two studies (Hedelin et al., 2000; Grandjean et al., 2004) reporting higher vagally-mediated HRV in boys.

While the overall analysis of both HF-HRV and RMSSD suggest lower vagal activity in girls, the inconsistencies in the effect sizes reported between HF-HRV and RMSSD might be associated with measurement techniques. Frequency-domain measures of HRV provide information of different quality and detail compared to time-domain measures (Sinnreich et al.,

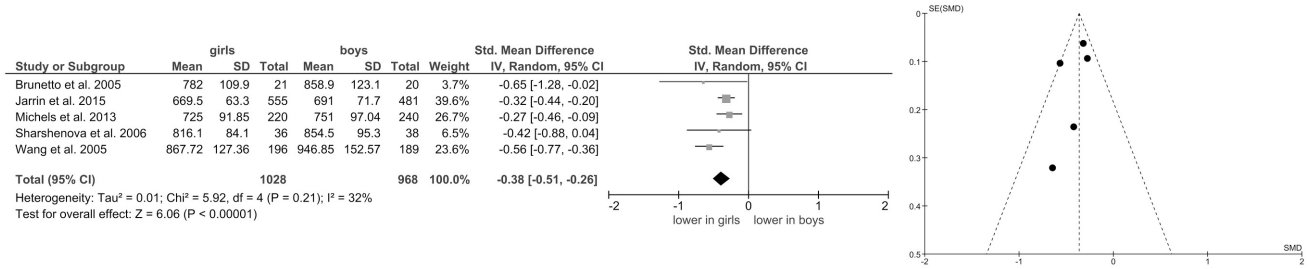

FIGURE 4 | Forrest- and Funnel-Plot from Meta-Analysis on Short-Term Resting State IBI; Brunetto et al. (2005): supine data used for analysis; Jarrin et al. (2015): data used as reported; Michels et al. (2013): data reported as mean and interquartile range, transformed before analysis; Sharshenova et al. (2006): supine data used for analysis; Wang et al. (2005): data pooled across ethnic groups.

1998). While RMSSD and HF-HRV are highly correlated (Goedhart et al., 2007), it has been suggested that time domain parameters can be estimated with less bias and variability then with frequency-domain parameters (Kuss et al., 2008). This issue relates, in part, to the influence of respiration on HF-HRV relative to RMSSD (Penttilä et al., 2001; Hill and Siebenbrock, 2009). The impact of respiration on HRV can be adjusted for during statistical modeling of short-term resting state HRV (Lewis et al., 2012), a procedure which is believed to yield a trait-like measure of vagal cardiac control that is relatively unaffected by situational constraints (Bertsch et al., 2012). Only two of the included studies on short-term recording controlled for respiration by instructing participants to breath in sequence with a metronome set at 15 breaths per minute (Brunetto et al., 2005) and 12 breaths per minute (Hedelin et al., 2000).

It is important to note that respiration rate decreases with increasing age (Fleming et al., 2011). In adults, the HF power band of the HRV power-spectrum is defined as the sum of the variance that occurs between 0.15 and 0.4 Hz. It is recommended that adjustment to these ranges be made for children and adolescents (Zisner and Beauchaine, 2017). All studies included in the present meta-analysis defined HF-HRV based on the frequency bands for adults (Hedelin et al., 2000; Grandjean et al., 2004; Gutin et al., 2005; Wang et al., 2005; Reed et al., 2006; Sharshenova et al., 2006; Greaves-Lord et al., 2007; Moodithaya and Avadhany, 2012; Michels et al., 2013; Koch and Pollatos, 2014; Jarrin et al., 2015). Given this lack of what is arguably most appropriate for HRV ranges (Fleming et al., 2011), future studies in children and adolescents should use time-domain measures of vagally-mediated HRV that are less affected by respiration, adjust frequency bands for spectral analysis or at least to record, and possibly control for respiration.

With respect to the focus of the Frontiers Research Topic, to "determined to what extent [...] alterations of HRV during growth and development result from [changes in HR]," we like to emphasize differences in the association of HRV and HR by age. Albeit meta-analytical findings prohibit causal interpretations in the absence of raw longitudinal data, there are notable difference and similarities in autonomic cardiac control comparing findings in adults (Koenig and Thayer, 2016) and the present results. In our previous meta-analysis reporting on various HRV parameters across age groups, we observed evidence that HF-HRV (in normalized units and log-transformed) is greater in women relative to men, but observed no sex effects for RMSSD (Koenig and Thayer, 2016). In meta-regression, we observed that RMSSD was not affected by respiration, but sex differences reported for HF-HRV were attenuated among studies that did not adjust for respiration. Most interestingly, we observed that age was a significant covariate on RMSSD —while meta-analysis on RMSSD showed no consistent effects, adult women displayed greater RMSSD compared to men with increasing age. The present analysis extends these results by demonstrating that girls have lower RMSSD at younger ages when compared to boys. Two of the included studies directly addressed age dependent differences between girls and boys (Grandjean et al., 2004; Moodithaya and Avadhany, 2012), with equivocal conclusions. Both studies reported that LF, HF, and total power components of HRV decreased from childhood to adolescents (Grandjean et al., 2004) and further into adulthood (Moodithaya and Avadhany, 2012). In one study this decline was more pronounced for boys than girls (Grandjean et al., 2004). Importantly, only one of these four studies used a prospective design that allowed for the evaluation of the same children at each time-point (Grandjean et al., 2004) whereas the other studies were cross-sectional. Most interestingly, independent of age, we found greater mean HR in adult women (Koenig and Thayer, 2016) and girls compared to their male counterparts of the same age. Based on these findings, we carefully suggest that the association of HR and HRV, that in itself is the topic of ongoing scientific debate (Sacha and Pluta, 2008; Pradhapan et al., 2014; Sacha, 2014; Gąsior et al., 2015), is sensitive to age.

Potential Mechanisms

Pubertal Development and Sex Hormones

There are many potential mechanisms underlying sex differences in resting state cardiac vagal activity in children and adolescents of which we will discuss two of the most prominent. While the pathophysiological mechanisms of altered vagal activity underlying psychopathology are not yet well-understood, the emerging influence of sex hormones during puberty may explain the observed differences between HF-HRV in boys and girls. A post-pubertal shift from lower vagal activity (pre-pubertal)

to greater vagal activity (post-pubertal) in girls compared to boys may be related to hormonal changes during this sensitive period of development. The female sex hormones estrogen and progesterone are associated with cardiac autonomic modulation and animal models indicate that estrogen enhances cholinergic muscarinic activity and has a facilitating effect on cardiac vagal function (Du et al., 1994). Changes in estrogen levels in girls associated with pubertal development may explain the shift from lower vagal activity in girls (pre-pubertal) to relative higher vagal activity in women. Further, human research evaluating the influence of menstrual cycle on cardiac autonomic modulation in women who are not using oral contraceptives have indicated that variations in female sex hormones that occur across the menstrual cycle influence cardiac vagal activity (Hirshoren et al., 2002; Vallejo et al., 2005; Bai et al., 2009; Tenan et al., 2014). These studies suggest that the early follicular phase (i.e., a time of low estrogen and progesterone) is characterized by increased cardiac vagal activity while the luteal phase (i.e., when estrogen and progesterone peak) is characterized by an increase in sympathetic activity. Moreover, the use of oral contraceptives to maintain constant estrogen and progesterone negate cardiac vagal differences observed throughout the menstrual cycle (Teixeira et al., 2015). The male androgens (in particular testosterone) might also account for the observed sex differences in cardiac vagal activity. Animal models have reported that the administration of androgen reduces cardiac vagal activity and this effect is reversed with the administration of an androgen antagonist (Marques Neto et al., 2013). Further, humans using androgenic-anabolic steroids have shown lower vagally-mediated HRV following exercise (Maior et al., 2013). Pubertal maturation and growth in boys that is associated with rising levels of androgens may shift the relative dominance of vagal activity to decreased vagal activity compared to girls in adulthood. Such an hypothesis fits current meta-theory on the association of sex-hormones, pubertal development and the development of sex-specific psychopathology (Martel, 2013). However, given that the sample of studies included in the meta-analysis comprised pre- and post-pubertal subjects, we were not able to address this question in greater detail. Future longitudinal studies are necessary to investigate the impact of pubertal maturation, associated changes in sex-hormones, and vagal activity.

Differences in Physical Activity

Greater physical activity in boys might underlie the effects observed in our present analysis. Boys are physically more active than girls (Trost et al., 2002; Sherar et al., 2007) and greater physical activity is associated with greater vagal activity (Goldsmith et al., 1997) while poor physical health in general (Kemp and Quintana, 2013) and less physical activity (i.e., Teisala et al., 2014) are associated with reduced vagal activity. Further, physical activity can increase vagally-mediated HRV (Rennie et al., 2003), in particular in preadolescents (Buchheit et al., 2007).

Most of the included studies on resting state HRV assessed physical activity/fitness. Participants in the study by Brunetto et al. (2005) completed a health questionnaire including questions on physical activity. Further, the authors determined oxygen consumption (VO^2) peak in an incremental exercise test and compared groups with low, mid, and high aerobic fitness. There were no group differences on RMSSD, or HF-HRV and the interaction of sex and aerobic fitness was not reported. Grandjean et al. (2004) used parent reports on physical fitness (much, average, or none) as a covariate in their analysis. The effect of physical activity on sex-differences in HRV was not reported. Gutin et al. (2005) measured physical activity with accelerometry and reported a positive association between physical activity and RMSSD. In their study boys reported significantly greater physical activity. Hedelin et al. (2000) used an incremental treadmill test with continuous analyses of VO^2 to determine physical fitness. Exact statistics were not reported, but descriptive data points to greater aerobic capacities in boys in this sample. Michels et al. used an accelerometer to monitor physical activity and used a Eurofit fitness test battery to determine maximal oxygen uptake. Boys were more active and physical fitness was associated with HRV in boys but not in girls. Moodithaya and Avadhany (2012) estimated the daily physical activity level based on a validated physical activity questionnaire, however physical activity did not differ between groups based on age and sex. Reed et al. (2006) controlled for aerobic fitness determined by a progressive shuttle run—but there were no sex differences. Thus, sex differences reported for short-term measures of HRV might relate to differences in physical activity/fitness reported in some studies.

Clinical Implications

We can only speculate if females gain or if males lose vagal activity during adolescence. However, it seems evident that while girls have lower vagal activity, women show greater vagal activity compared to their male counterparts and independent of age show greater mean HR. With respect to the development of psychopathology, in particular during the sensitive period of adolescence, this result has important implications. Vagal activity during a resting state is a potential biomarker of clinical relevance with respect to diagnosis, monitoring, and treatment of psychopathology. For example, reduced resting state HF-HRV is both a correlate of depression among adults (e.g., Kemp et al., 2010), and a marker of treatment response (Chambers and Allen, 2002; Chien et al., 2015) such that decreases in depressive symptoms are associated with increases in HF-HRV. Furthermore, stimulation of the vagus nerve, originally developed for the treatment of epilepsy, is a promising approach for addressing treatment refractory depression among adults (Groves and Brown, 2005; O'Reardon et al., 2006) and may have potential applicability in younger populations. Furthermore, findings from the present review and meta-analysis have implications for somatic ill-health and CVD in particular. Longitudinal studies are warranted, addressing the predictive value of sex-differences in vagal activity in children and adolescents in the development of other somatic disorders, including CVD in adulthood.

CONCLUSION

Drawing on data from about 5,000 children and adolescents, we provide evidence that healthy young girls display lower

resting-state vagally-mediated HRV compared to boys. These sex differences in cardiac autonomic function may represent a potential pathophysiological mechanism contributing to sex-differences in psychopathology. Longitudinal studies across pubertal development are needed to support this claim by empirical evidence.

AUTHOR CONTRIBUTIONS

JK and JR conducted the literature research and analysis. JK and JR wrote the first draft of the manuscript. TC, JT, and MK provided important intellectual content in revising the manuscript. All authors provided final approval before submission.

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Influence of Heart Rate in Non-linear HRV Indices as a Sampling Rate Effect Evaluated on Supine and Standing

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The purpose of this study is to characterize and attenuate the influence of mean heart rate (HR) on nonlinear heart rate variability (HRV) indices (correlation dimension, sample, and approximate entropy) as a consequence of being the HR the intrinsic sampling rate of HRV signal. This influence can notably alter nonlinear HRV indices and lead to biased information regarding autonomic nervous system (ANS) modulation. First, a simulation study was carried out to characterize the dependence of nonlinear HRV indices on HR assuming similar ANS modulation. Second, two HR-correction approaches were proposed: one based on regression formulas and another one based on interpolating RR time series. Finally, standard and HR-corrected HRV indices were studied in a body position change database. The simulation study showed the HR-dependence of non-linear indices as a sampling rate effect, as well as the ability of the proposed HR-corrections to attenuate mean HR influence. Analysis in a body position changes database shows that correlation dimension was reduced around 21% in median values in standing with respect to supine position ($p < 0.05$), concomitant with a 28% increase in mean HR ($p < 0.05$). After HR-correction, correlation dimension decreased around 18% in standing with respect to supine position, being the decrease still significant. Sample and approximate entropy showed similar trends. HR-corrected nonlinear HRV indices could represent an improvement in their applicability as markers of ANS modulation when mean HR changes.

Keywords: HRV, ANS, HR-correction, nonlinear, entropy, D_2, sampling rate

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INTRODUCTION

Heart rate (HR) variability (HRV) has been studied as a non-invasive technique to assess autonomic nervous system (ANS) regulation of the heart. Although, HRV analysis is still controversial (Karemaker, 2006), its content has been related to sympathetic and parasympathetic modulation by Task Force of the ESC-NASPE (1996) and Sassi et al. (2015).

During the last decades, HRV analysis has been extended including nonlinear indices based on chaos theory. These methodologies describe ANS in terms of regularity and complexity. Non-linear

indices have been studied in a wide range of cardiovascular diseases revealing discriminant power for risk stratification (Maestri et al., 2007). Correlation dimension was used to stratify women who suffered hypotension during spinal anesthesia in cesarean section (Chamchad et al., 2004; Bolea et al., 2014a). Sample and approximate entropy were studied comparing control vs. children with congenital heart malformation due to effects of cyanotic and acyanotic defects (Aletti et al., 2012). Furthermore, the integration of linear and nonlinear HRV indices has been shown relevant to stratify cardiac risk patients (Voss et al., 2009) and to describe pathophysiological mechanisms in the cardiovascular and neural system control (Signorini et al., 2011). Some studies pointed out that HRV complexity changes as a result of sympathetic activation (Porta et al., 2007; Turianikova et al., 2011; Weippert et al., 2013).

However, the physiological interpretation of HRV as a marker of ANS activity may be blurred since several factors could affect how intrinsic pacemaker cells and ANS activity are expressed in HRV (Yaniv et al., 2014a). The nonlinear relationship between temporal and complexity HRV indices with respect to HR has been addressed emphasizing the importance of attenuating this effect (Zaza and Lombardi, 2001; Platisa and Gal, 2006; Monfredi et al., 2014; Yaniv et al., 2014b). Furthermore, different mathematical models have demonstrated a relationship between HRV amplitude and HR correcting it (Chiu et al., 2003; Meste et al., 2005; Bailón et al., 2011; Sacha, 2014; Billman et al., 2015). HR correction effect on HRV analysis was studied to predict risk mortality (Pradhapan et al., 2014). Non linear indices, such as correlation dimension, sample, and approximate entropy, are computed over linearly detrended and normalized series so this effect is already compensated for (Osaka et al., 1993; Pincus et al., 1993; Porta et al., 2007; Voss et al., 2009; Bolea et al., 2014a). Despite this normalization, HR may still influence nonlinear HRV indices due to the fact that HR is the intrinsic sampling rate of HRV signal. This implies that the amount of information captured during the same time interval depends on HR. The dependence of nonlinear indices on data length has already been reported (Havstad and Ehlers, 1989), and some studies have computed nonlinear HRV indices over interpolated RR time series at different sampling rates to increase index reliability (Theiler, 1990; Osaka et al., 1993; Hagerman et al., 1996; Radhakrishna et al., 2000; Javorka et al., 2002; Kim et al., 2005). Our hypothesis is that the influence of HR as sampling rate on nonlinear HRV indices is still noticeable even when the same data length is considered.

The goal of this study is to assess and attenuate the HR influence as sampling rate on nonlinear HRV indices in order to provide insight in their physiological interpretation as markers of ANS modulation. To assess the influence of HR on nonlinear HRV indices, a simulation study is conducted in which changes in ANS modulation are independent of changes on mean HR. Based on simulation results, two approaches are proposed to attenuate this mean HR influence. Finally, HR-corrected nonlinear HRV indices are computed over a body position changes database to study their performance under ANS elicitation.

MATERIALS

Body Position Changes (BPC) Database

This database was developed collaboratively at Harvard Medical School, Massachusetts Institute of Technology, and the Favaloro Foundation Medical School. The whole cohort of short-term recordings comes from two data collecting studies. Further details of this database can be found in Sobh et al. (1995).

First Study

Thirteen male subjects of age 21.6 $\pm$ 4.4 years (Mean $\pm$ SD; range, 19–38 years) with no history of cardiopulmonary disease participated in a study carried out at Clinical Research Center at the Massachusetts Institute of Technology, USA.

Second Study

It comprises groups of subjects of different ages. Only the young group was included in our work (9 subjects, 26.7 $\pm$ 4.7 years; range, 20–35 years).

Thus, from the whole database we selected 22 subjects. Two recordings per subject were acquired containing 7-min electrocardiographic (ECG) and respiration (RP) signals, sampled at 360 Hz. The protocol included postural changes. First, ECG and RP signals were recorded while subjects were in supine position. Then, subjects changed to standing position and after 5 min, to allow reaching hemodynamic equilibrium, ECG and RP signals were recorded in standing position. Subjects were asked to breathe following an irregular sequence of tones (Sobh et al., 1995).

Fantasia Database

Twenty young rigorously-screened healthy subjects underwent 120 min of supine resting while continuous ECG and RP signals were recorded at 250 Hz while watching the movie Fantasia, Disney 1940, to help maintain wakefulness. Further database information is available elsewhere (Iyengar et al., 1996) and can be downloaded from http://www.physionet.org (Goldberger et al., 2000).

METHODS

ECG Preprocessing

Because the reliability of the HRV analysis can be compromised by low sampling frequency of ECG recordings (Merri et al., 1990), the ECGs belonging to BPC and Fantasia database were interpolated by cubic splines to a frequency of 1080 and 1000 Hz, respectively. Then, heartbeat times, $t(k)$ where k symbolizes the k^{th} beat, were estimated using an ECG wavelet-based detector (Martínez et al., 2004). Ectopic beats were identified imposing a time-varying threshold on instantaneous heart rate variations. Then, these ectopic beats are corrected using the IPFM model, as described in Mateo et al. (Mateo and Laguna, 2003).

Non-linear HRV Analysis

Approximate, sample entropy and correlation dimension are methods that exploit the phase-space representation of a time series based on Taken's theorem (Takens, 1981). These nonlinear

methods are described in the following and further mathematical details are provided in Appendix.

Approximate and Sample Entropy

SampEn and *ApEn* are irregularity measurements of the time series (Pincus, 1995). Although both entropies are closely related to each other, *SampEn* was introduced to overcome the self-pairs-related limitation of *ApEn* computation. Briefly, patterns of time series values (reconstructed vectors) of a certain length (embedded dimension, m) are compared to the rest of the possible pattern candidates. Those comparisons whose differences are below a threshold (r) are summed up and used to calculate correlation sums. The final entropy value measures the changes produced when increasing the length of the patterns in one unit. The parameters m and r have to be previously defined to estimate the entropy values. In this work parameter values are set to $m = 2$ and $r = 0.15$ for *SampEn*. For *ApEn*, $m = 2$ and r is set as the threshold that maximizes approximate entropy ($ApEn_{\max}$; Yentes et al., 2013). $ApEn_{\max}$ was selected instead of *ApEn* to avoid the bias introduced by in *ApEn* when considering self-comparisons (Mayer et al., 2016). Its computation is based on a previously published algorithm (Bolea et al., 2014a).

Correlation Dimension

Correlation dimension, D_2, measures the degree of complexity of the system that generates the time series (Grassberger and Procaccia, 1983). In a previous work, we developed techniques to improve the estimation of correlation dimension (Bolea et al., 2014a). On that study log-log curves (logarithm of correlation sums vs. logarithm of thresholds) were fitted to sigmoid curves, thus increasing the accuracy of maximum slope estimation. Moreover, another estimate of correlation dimension denoted as $D_{2(max)}$ based on the points that maximize the difference between each pair of sigmoid curves was presented. Both D_2 and $D_{2(max)}$ were computed by varying $m = 1$–16 and $r = 0.01$–3 in steps of 0.01.

Non-linear indices estimation may be compromised when the amplitude value of time series appears discrete in a reduced set of values due to the lack of variation. A pre-processing stage is included and details can be found elsewhere (Bolea et al., 2014a).

Simulation Study

A simulation study is conducted to assess the mathematical relationship between HR and nonlinear HRV indices. The simulation study was carried out based on a HRV representation through the IPFM model. This model assumes that the ANS influence on the sinoatrial node can be represented by a modulating signal, $\mathcal{M}(t)$ (Mateo and Laguna, 2000). According to this model, when the integral of $1+\mathcal{M}(t)$ reaches a threshold, T, a new heartbeat is generated at time instant $t(k)$. Threshold T represents the inverse mean HR.

Fantasia database was selected to compute modulating signals. Assuming that $\mathcal{M}(t)$ is causal, band-limited and $\mathcal{M}(t) < 1$ then the instantaneous HR can be described as:

$$d_{HR}(t) = \frac{1 + \mathcal{M}(t)}{T} \quad (1)$$

Instantaneous heart rate $d_{HR}(t)$ is obtained from the heartbeat times $t(k)$ based on the IPFM model (Mateo and Laguna, 2003), and sampled at 4 Hz. A time-varying mean heart rate $d_{HRM}(t)$ is computed by low pass filtering $d_{HR}(t)$ with a cut-off frequency of 0.03 Hz. The heart rate variability signal is obtained as $d_{HRV}(t) = d_{HR}(t) - d_{HRM}(t)$. Finally, the modulating signal, $\mathcal{M}(t)$, is approximated by $d_{HRV}(t)/\overline{d_{HRM}(t)}$ (Bailón et al., 2011), that is the HRV signal corrected or normalized by the mean HR.

Spectral analysis was applied to 5-min modulating signals $\mathcal{M}(t)$ by Welch periodogram. Frequency domain indices were estimated based on spectral bands (LF band from 0.04 to 0.15 Hz and HF band from 0.15 to 0.4 Hz). Respiratory frequency was checked to be within the HF band.

Among all modulating signals, only those which presented one marked peak on each band (LF and HF band) were selected. Spectral indices such as the powers and the frequency peaks were used to generate synthetic modulating signals using an autoregressive moving average technique (ARMA; Orini et al., 2012). A total of one hundred 5-min segments were selected and their spectral indices were used to feed the ARMA model. A total of $M = 50$ stochastic modulating signals $\mathcal{M}^{j}(t)$ with $j = 1,...,M$, were simulated for each $\mathcal{M}(t)$. **Figure 1** shows the spectra of 50 stochastic realizations, their median spectrum and the one of the segment-recording they are based on.

Then, the IPFM model was applied on each stochastic realization, varying the parameter T_n, where $n = 1,...,16$, corresponding to T from 0.46 to 1.1 s in 0.04 s steps, to simulate the heartbeat occurrence times, $t^{j}_{Tn}(k)$, from which simulated 300-sample, are obtained. In this way, simulated RR series are generated where ANS modulation is independent from changes in mean HR. Simulation scheme is illustrated in **Figure 2**. D_2, *SampEn* and $ApEn_{(max)}$ are computed over these simulated RR time series.

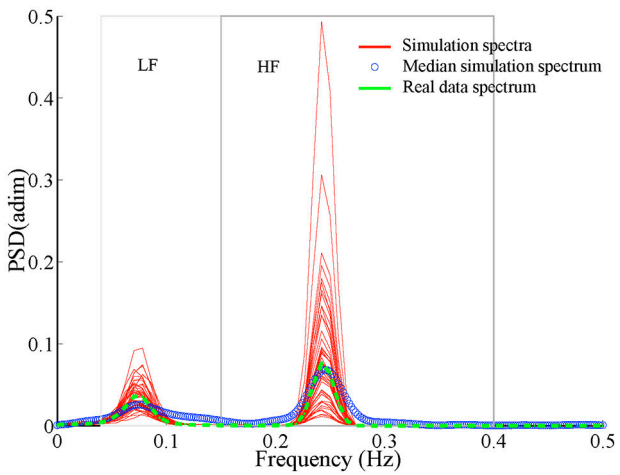

FIGURE 1 | Spectra derived from 50 stochastic realizations of simulated modulating signals during supine conditions (data simulates subject conditions from Fantasia database) applying ARMA technique fixing LF and HF content (red lines). Average spectrum is shown in circles (blue) and spectrum belonging to real data in dashed line (green).

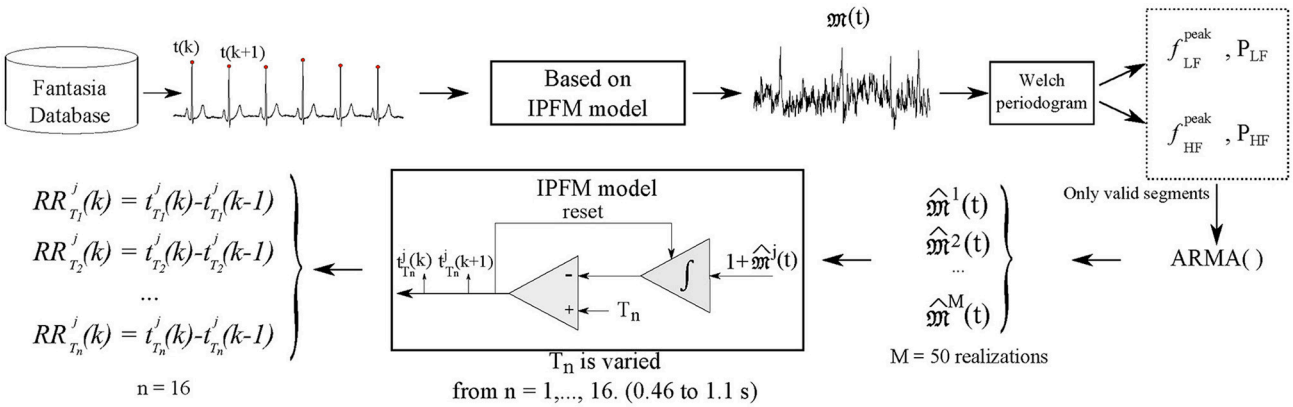

FIGURE 2 | Simulation data scheme is illustrated. Heartbeat time occurrences are detected from ECG. Based on the IPFM model the ANS modulating signal $\mathfrak{M}(t)$ is estimated. Spectral analysis by Welch periodogram is computed on $\mathfrak{M}(t)$ in order to estimate the parameters needed for the simulation, frequency and power of LF and HF components, which are used to construct a new set of modulating signals, $\mathfrak{M}^j(t)$, through ARMA technique, with $M = 50$ realizations. Then, IPFM model is used to generate simulated heartbeat occurrences $t^j_{Tn}(k)$ with different values of T from 0.46 to 1.1 s. Simulated RR series are computed from the $t^j_{Tn}(k)$.

Another simulation was done based on the BPC database characteristics. However, since subjects were asked to breathe following an irregular sequence of tones, the HF band does not show a dominant peak. In this case, the low and high frequencies used to feed the ARMA model were placed in the middle of LF and HF band, respectively. Then, modulating signals were simulated from spectral indices derived from supine and upright positions. This extends the analysis of HRV dependence on HR under enhanced sympathetic conditions.

Non-linear Indices Dependence on HR as Sampling Rate

The methodology used to compute nonlinear HRV indices, considered in this study, is applied over linearly detrended and normalized RR time series. The detrending ensures that mean HR values are removed from the series whereas the normalization eliminates the influence of mean HR on HRV amplitude. Despite this fact, the effect of mean HR as sampling rate might still be present on them. In this section this effect is investigated on the simulation study, where changes in mean HR are independent from changes in ANS modulation. First, a mathematical relationship between nonlinear HRV indices and HRM is assessed by two regression formulas; then, a HR-correction is proposed based on these formulas. Second, interpolation of RR series is proposed to attenuate the sampling rate influence by mean HR on nonlinear HRV indices.

Regression Formulas

In order to explore the relationship between nonlinear HRV indices and HR the following regression models were proposed.

$$\mathrm{X} = \beta + \alpha RR, \text{(Linear model)}, \tag{2}$$

$$\mathrm{X} = \beta(RR^{\alpha}), \text{(Parabolic model)}, \tag{3}$$

where $\mathrm{X} \in \{D_2, SampEn, ApEn\}$, α and β are regression coefficients.

Based on the former models HR-correction formulas were obtained by projecting each nonlinear index onto a standard level of $RR = 0.5$ s, hence:

$$\text{Linear}: \mathrm{X}_{C1} = \mathrm{X} + \xi(0.5 - \mathrm{RR}), \tag{4}$$

$$\text{Parabolic}: \mathrm{X}_{C2} = \mathrm{X}\left(\frac{0.5}{\mathrm{RR}}\right)^{\xi}, \tag{5}$$

where ξ is the correction factor.

Transformation of X_{C1} or X_{C2} and RR into linear relationship was used to compute Pearson correlation coefficient ρ. Then, optimization was assessed by total least squares providing correction factors by the Golden Cut Search satisfying $\rho(\xi) = 0$.

Correction factors were computed on each stochastic realization. Thus, subject-specific correction was defined considering the correction factors of the 50 stochastic realizations for each modulating signal and computing the median of the HR-corrected indices.

Furthermore, a unique correction parameter was computed considering all stochastic realizations for all modulating signals. The transformation and optimization technique described above was applied to median values for each nonlinear index, thus defining a median correction approach to obtain $X^{\xi}{}_{C1}$ and $X^{\xi}{}_{C2}$.

Interpolation

RR series are unevenly sampled being the HR its sampling rate. This implies that the number of data information for the same time interval is dependent on HR. On the other hand, it is known that estimation of nonlinear indices such as correlation dimension, sample, and approximate entropy are dependent on data length (Havstad and Ehlers, 1989). Therefore, interpolating RR time series at the same sampling rate may alleviate the influence of mean HR on nonlinear HRV indices since it allows using the same number of data for the same time interval. Interpolation at 2, 4, and 8 Hz were studied (X_{I2}, X_{I4}, and X_{I8} respectively).

Statistical Analysis

Kolmogorov–Smirnov test was used to test the normality of data distributions. Mann–Whitney *U*-test was used when necessary; otherwise paired *T*-test was applied. Furthermore, Pearson correlation was used to assess linear correlation between corrected nonlinear HRV indices and *RR*. $p < 0.05$ are considered as statistically significant.

Bland–Altman plots were used to analyse the agreement of subject-specific vs. median correction formulas. The intra-classes coefficient (ICC) was computed by SPSS for Windows, Version 15.0. Chicago, SPSS Inc.

RESULTS

Non-linear HRV Indices and Mean HR Reflect Body Position-Induced Changes

Non-linear HRV indices (D_2, *SampEn*, and $ApEn_{\max}$) were computed for the BPC database considering 300-sample segments. All of them were found significantly higher in supine than in standing position (see **Figure 3B**). Mean HR was also significantly higher in supine than in standing position (**Figure 3A**), which might explain the statistical differences observed in the computed nonlinear HRV indices.

Relationship between Non-linear HRV Indices and RR in the Simulation Study

The relationship between nonlinear HRV indices and RR is assessed in the simulation study where RR is changed without changes in ANS modulation. Non-linear HRV indices computed from simulated data are illustrated in **Figure 4** (median values shown as blue circles). The correlation of nonlinear indices and mean HR was evaluated by Pearson correlation coefficient finding high correlation, for a wide range of median index values being 3.5–5.02 for $D_{2(\max)}$, 0.42–1.02 for *SampEn*, and 0.72–1.24 for $ApEn_{\max}$ (see **Table 1**).

HR-Corrected Non-linear Indices by Regression Formulas

Regression formulas were applied to each simulated modulating signal (subject-specific approach) providing corrected indices with minimal mean HR correlation. The obtained HR-corrected nonlinear indices are shown in **Figure 4** (median values considering all segments, in triangles right and left for linear and parabolic regressions, respectively). The application of correction formulas alleviated the correlation between nonlinear indices and mean HR. Furthermore, the range covered by them was highly reduced (3.81–3.95 for $D_{2(max)}$, 0.57–0.61 for *SampEn*, and 0.8–0.9 for $ApEn_{max}$), see **Table 1**.

A set of correction factors (median approach) was obtained by considering the median of all nonlinear index values for each heart rate and computing global correction parameters (**Table 1**). To evaluate the agreement between subject-specific vs. median correction approaches, the ICC was analyzed, being above 0.8 for all HR-corrected nonlinear indices and for both proposed regression formulas. The Bland–Altman plot in **Figure 5** illustrates the difference between both approaches in which $D_{2(max)}$ is shown as an example.

HR-Corrected Non-linear Indices by Interpolation

The nonlinear indices were computed from simulated RR time series resampled at 2, 4, and 8 Hz. As shown in **Figure 4**, the corrected nonlinear index values obtained applying regression formulas projected onto $RR = 0.5$ s and by interpolating RR time series at 2 Hz have similar median values. Despite the fact that Pearson correlation factor computed between HR-corrected nonlinear HRV indices by interpolation and mean HR is still significant their range is much reduced [3.84–3.86 for $D_{2(max)}$, 0.588–0.592 for *SampEn*, and 0.824–0.836 for $ApEn_{max}$] being negligible compared to the range of uncorrected nonlinear ones.

Application to BPC Database

The proposed HR-corrections were evaluated in the BPC database. The results shown in **Figure 3C** illustrate the differences found between supine and standing conditions. Median and interquartile range of uncorrected and HR-corrected nonlinear HRV indices are provided in **Table 2**.

In a first study, the value of the median correction factor ξ extracted from the simulation study was used. It is worth noting that after linear correction there was no significant difference in *SampEn* and $ApEn_{max}$ between supine and standing positions, while parabolic correction only reduced differences below significance for *SampEn*.

In a second study, simulation of each recording's characteristics was computed to apply subject-specific correction, derived independently from supine, and standing recordings. HR-corrected $D_{2(max)}$ was found statistically significantly different for linear and parabolic regression formulas whereas $ApEn_{max}$ was only significant for parabolic.

Finally, nonlinear HRV indices were computed on RR time series interpolated at 2, 4, and 8 Hz. We can conclude that the higher the interpolation order, the lower the nonlinear HRV values. In all cases HR-corrected nonlinear indices calculated by interpolation showed statistical differences between positions regardless of the interpolation order used (results of 4 and 8 Hz not included in the manuscript) being their range notably reduced.

DISCUSSION

HRV analysis has been widely used as a non-invasive technique to assess and quantify cardiac ANS modulation (Task Force of the ESC-NASPE, 1996; Voss et al., 2009; Sassi et al., 2015). However, HRV analysis is still under investigation due to HRV characteristics that could lead to physiological misinterpretations (Osaka et al., 1993; Chiu et al., 2003). ANS modulation is linked to ANS tone (HR mean) and, as a consequence, an increase in the sympathetic activity and a decrease in the vagal tone are related to an increment in the HR and a reduction on its variability (Chiu et al., 2003; Kazmi et al., 2016). This implies that there is a physiological correlation between HRV and HR. However,

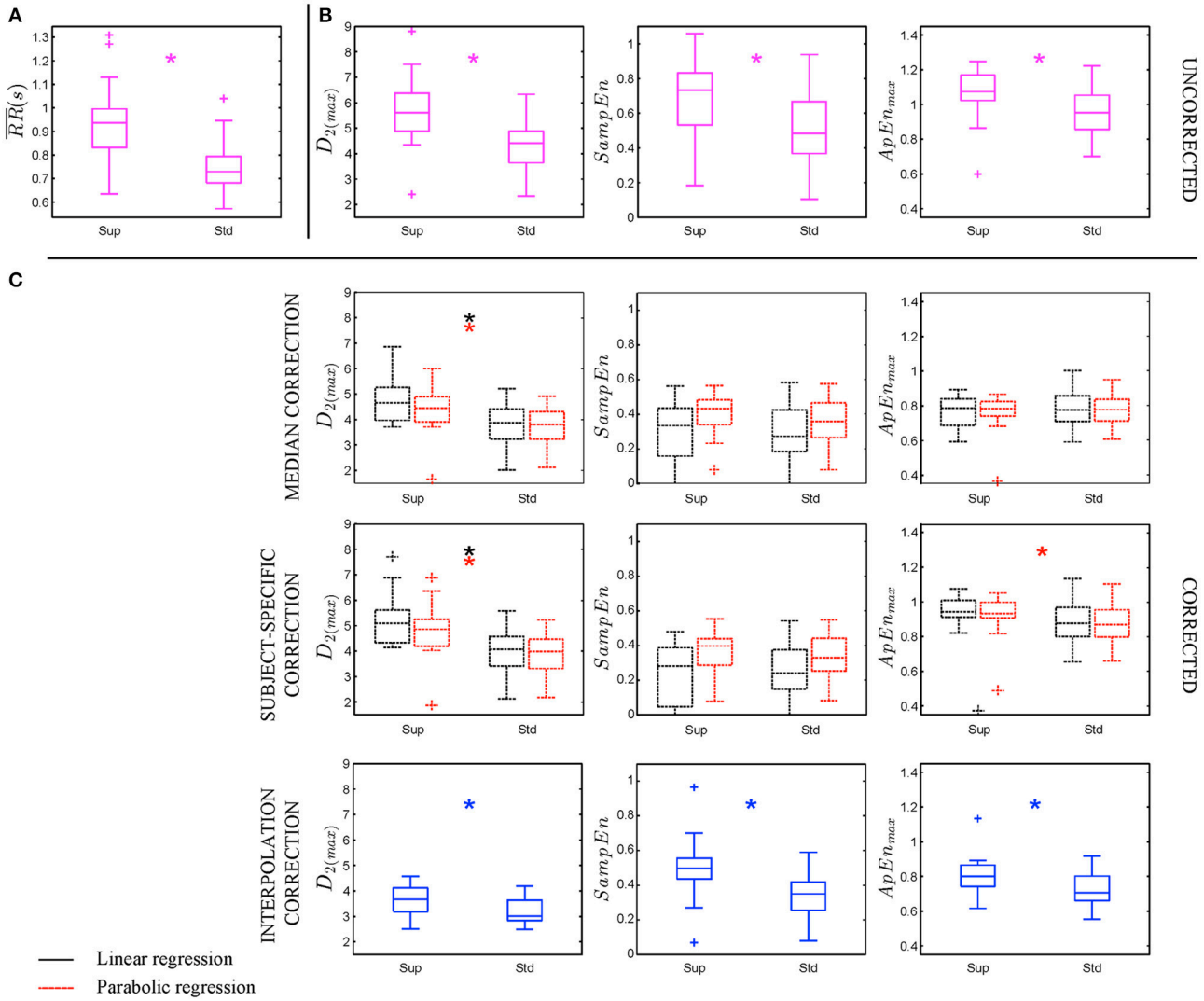

FIGURE 3 | BPC database (22 subjects) was analyzed in which supine and standing positions were compared. (A,B) Illustrate mean RR and uncorrected nonlinear HRV indices while **(C)** shows HR-corrected nonlinear HRV indices. Asterisks (*) indicates $p < 0.05$ by Mann–Whitney *U*-test between supine (Sup) and standing (Std) positions.

we have demonstrated that there exists also a methodological influence between nonlinear HRV indices and HR due to the fact that HR is the intrinsic sampling rate of HRV. To do this, we have conducted a simulation study. ANS modulating signals were generated as realizations of a stochastic process (Orini et al., 2012). Then, heart beat occurrences are generated using an IPFM model, which is based on action potential generation in SA node cells, and has been proven appropriate to describe the genesis of HRV (Mateo and Laguna, 2000). This simple model allows keeping the ANS modulation constant for different mean HR values, which is not possible in the reality. This simulation allowed assessing the nonlinear dependence of the standard deviation of normal beats on mean HR as it has been pointed out in previous studies (Zaza and Lombardi, 2001; Monfredi et al., 2014; Yaniv et al., 2014b; data not included in the manuscript). Two approaches have been proposed to attenuate the effect shown in the simulation study: regression formulas and interpolation.

Regression Formulas

Regression formulas are commonly used to characterize the relationship between two magnitudes such as ventricular repolarization and heart rate (Pueyo et al., 2004; Smetana et al., 2004; Baumert et al., 2010). The relationship between correlation dimension, sample, and approximate entropy, computed over simulated 300-sample RR series, and mean HR was studied by linear and parabolic regression. Although, the use of other regression models different from linear or parabolic ones may suppose an improvement, it would be unjustified since coefficients of determination $R^2 \geq 0.9$ were obtained for all cases for these models. Then, a correction was proposed based on regression formulas derived for each simulated case, the

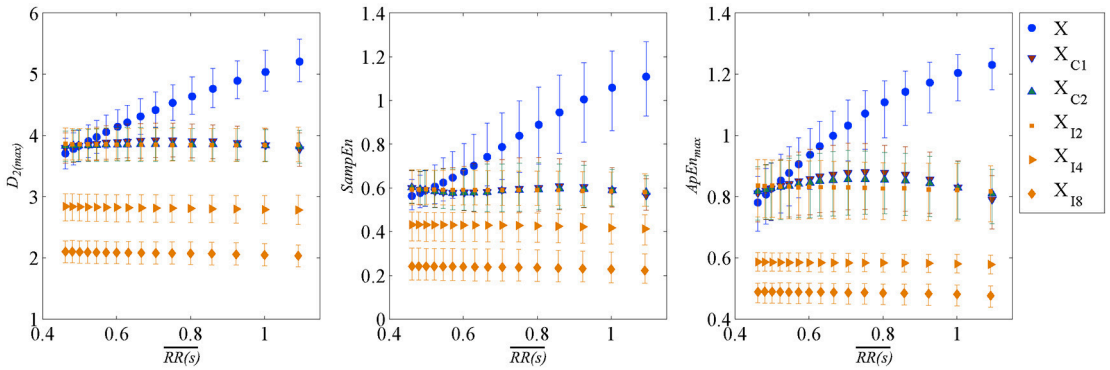

FIGURE 4 | Non-linear HRV indices computed from simulation study varying mean heart period, distribution of all uncorrected nonlinear HRV indices are shown in (blue) circles, corrected by linear regression in (brown) down triangles, corrected by parabolic regression in (green) up triangles, both former cases only subject-specific correction value of non-linear indices for each RR were shown. Finally, HR-corrected non-linear indices by interpolating RR time series at 2, 4, and 8 Hz in (orange) diamonds. Data corresponds to all simulations derived from Fantasia database. Distributions are represented by median and interquartile range.

TABLE 1 | Pearson correlation factor ρ and *p*-values of non-linear indices and RR obtained from simulation study.

	$D_{2(max)}$	SampEn	$ApEn_{max}$
ρ	0.959 ± 0.068	0.947 ± 0.1	0.949 ± 0.074
p-value	0.0002 ± 0.0015	0.0004 ± 0.0044	0.0003 ± 0.0022
Median ± IQR	4.26 ± 0.76	0.72 ± 0.30	0.98 ± 0.26

Regression formulas	**Linear**			**Parabolic**		
	$D_{2(max)C1}$	$SampEn_{C1}$	$ApEn_{C1}$	$D_{2(max)C2}$	$SampEn_{C2}$	$ApEn_{C2}$
R^2	0.919 ± 0.129	0.896 ± 0.186	0.902 ± 0.139	0.923 ± 0.129	0.896 ± 0.179	0.910 ± 0.125
ρ sub-spe ($\times 10^{-05}$)	0.013 ± 0.20	0.016 ± 0.21	−0.0051 ± 0.20	−0.016 ± 0.20	0.011 ± 0.20	0.01 ± 0.20
p-value $_{sub-spe}$	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Median , IQR	3.88 ± 0.07	0.59 ± 0.02	0.85 ± 0.04	3.85 ± 0.02	0.59 ± 0.01	0.84 ± 0.03
R^2_{Median}	0.997	0.988	0.970	0.999	0.990	0.982
ρ_{Median} ($\times 10^{-05}$)	−0.787	−0.109	−0.051	0.661	0.232	0.061
p-value $_{Median}$	1	1	1	1	1	1
ξ Correction factor	2.39	0.93	0.75	0.39	0.84	0.54
Median ± IQR	3.88 ± 0.07	0.59 ± 0.02	0.85 ± 0.05	3.85 ± 0.02	0.59 ± 0.01	0.84 ± 0.03

2 Hz Interpolation	$D_{2(max)}$	SampEn	$ApEn_{max}$
ρ_{I2}	−0.473 ± 1.41	−0.39 ± 1.09	−0.29 ± 0.77
p-value $_{I2}$	0.0005 ± 0.044	0.008 ± 0.19	0.068 ± 0.37
Median ± IQR	3.85 ± 0.01	0.59 ± 0.002	0.83 ± 0.006

Linear and parabolic dependence of non-linear indices on RR was evaluated. Coefficient of determination (R^2) as well as Pearson correlation factor and p-values (considering subject-specific and median correction) and ξ correction factor are presented for both regressions. Pearson correlation factor ρ and p-values of non-linear indices and RR obtained interpolating the simulated RR time series at 2 Hz. Data are given as median ± interquartile range.

so-called subject-specific correction, minimizing nonlinear HRV indices correlation to mean HR. A correction based on regression formulas derived from median parameters was proposed as an extension to be applied to other databases. ICC values >0.8 were found when evaluating subject-specific vs. median correction approaches for all nonlinear HRV indices, suggesting the usage of either of the approaches, see **Figure 5**.

Interpolation

Simulated RR time series were interpolated at 2, 4, and 8 Hz. The higher the interpolation rate, the lower the nonlinear index values. The addition of new data, resulting from interpolation, can be interpreted in terms of entropy as an increase in signal regularity being in concordance with a previous work in which electroencephalogram complexity through correlation

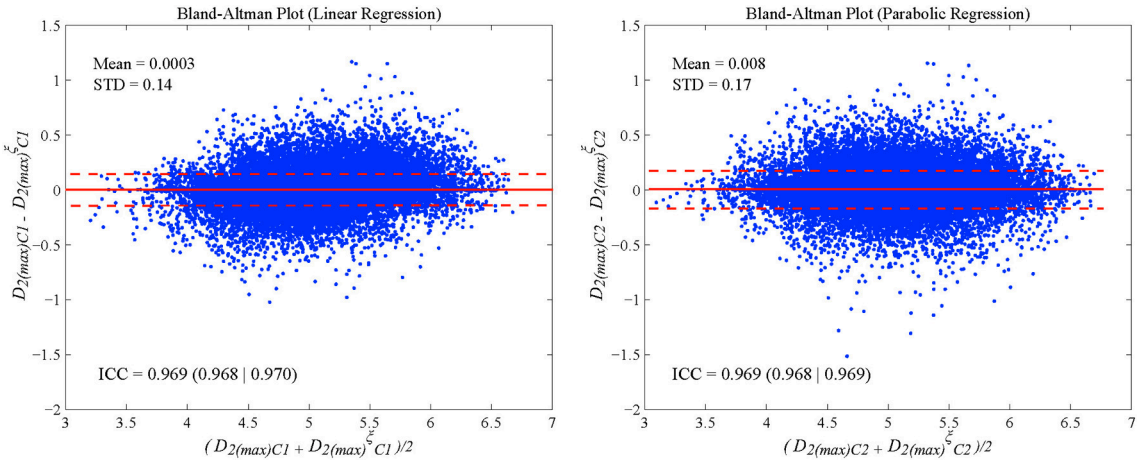

FIGURE 5 | Bland-Altman plots and intra-class coefficient (ICC) illustrate the agreement between subject-specific and median correction approaches computed on Fantasia database to correct HR effect for both proposed regressions for corrected $D_{2(max)}$ index: linear (left panel) and parabolic (right panel).

TABLE 2 | Uncorrected Non-linear HRV indices and HR corrected by proposed approaches.

	Supine	Standing	*p*-value
$D_{2(max)C1}$	5.61 (4.88|6.38)	4.41 (3.64|4.88)	0.0025
$D_{2(max)C1}$	5.10 (4.33|5.62)	4.07 (3.41|4.57)	0.0014
$D_{2(max)C2}$	4.85 (4.19|5.25)	3.97 (3.31|4.46)	0.0019
$D_{2(max)C1}^{\xi}$	4.66 (3.98|5.27)	3.88 (3.24|4.42)	0.0045
$D_{2(max)C2}^{\xi}$	4.47 (3.93|4.93)	3.82 (3.25|4.32)	0.0064
$D_{2(max)I2}$	3.67 (3.23|4.08)	3.02 (2.84|3.64)	0.024
SampEn	0.73 (0.53|0.83)	0.48 (0.037|0.0.67)	0.008
$SampEn_{C1}$	0.28 (0.05|0.38)	0.24 (0.15|0.37)	0.73
$SampEn_{C2}$	0.40 (0.28|0.44)	0.33 (0.25|0.44)	0.44
$SampEn_{C1}^{\xi}$	0.333 (0.158|0.434)	0.272 (0.185|0.424)	0.39
$SampEn_{C2}^{\xi}$	0.436 (0.344|0.488)	0.364 (0.27|0.47)	0.062
$SampEn_{I2}$	0.50 (0.42|0.54)	0.35 (0.26|0.42)	0.0013
$ApEn_{(max)C1}$	1.11 (1.03|1.17)	0.88 (0.77|0.95)	0.008
$ApEn_{(max)C1}$	0.94 (0.91|1.01)	0.88 (0.80|0.97)	0.057
$ApEn_{(max)C2}$	0.94 (0.91|0.99)	0.87 (0.79|0.96)	0.038
$ApEn_{(max)C1}^{\xi}$	0.784 (0.684|0.838)	0.775 (0.707|0.856)	0.5
$ApEn_{(max)C2}^{\xi}$	0.783 (0.742|0.825)	0.777 (0.713|0.838)	0.94
$ApEn_{(max)I2}$	0.80 (0.74|0.85)	0.71 (0.66|0.80)	0.0098

Data is shown in terms of median and interquartile range. Statistical differences are tested by Mann–Whitney U-test.

dimension was evaluated varying the sampling rate (Jing and Takigawa, 2000). In our study, interpolation was used as a technique to alleviate the dependence of nonlinear HRV indices on mean HR as sampling rate effect since it allows estimating nonlinear indices over the same time interval and the same number of points. Sampling rate should be above the maximum HR. HR-correction nonlinear HRV indices computed by interpolating at 2 Hz and by regression formulas presented similar values and range. In some studies, RR time series were interpolated to increase the number of data points to increase reliability of nonlinear measurements, thus compensating mean HR effect on them. The used sampling frequency varies including 2, 4, and 8 Hz, or even 20 KHz (Osaka et al., 1993; Hagerman et al., 1996; Kim et al., 2005). However, since nonlinear HRV indices estimates are strongly dependent on the selected sampling rate, results should be compared with caution.

Despite the dependence of nonlinear HRV indices on mean HR revealed in the simulation study, no HR-correction of nonlinear HRV indices is applied in most of the studies found in the literature, where mean HR values are even not provided in some cases (Penttilä et al., 2003; Platisa and Gal, 2006; Melillo et al., 2011; Kunz et al., 2012; Moon et al., 2013; Weippert et al., 2013). The application of nonlinear indices without HR correction should be restricted to HR steady-state group conditions, as for example in Weippert et al. (2013).

Classical nonlinear HRV indices evaluated in the BPC database showed around 21, 34, and 21% of reduction in median values from standing with respect to supine position for $D_{2(max)}$, *SampEn*, and $ApEn_{max}$ respectively while mean HR increased by around 28%. Changes in these indices may reflect changes in mean HR as well as additional changes in ANS modulation, as suggested in a previous study (Platisa and Gal, 2006; Yaniv et al., 2014a).

In the BPC database, HR-corrected nonlinear indices were computed under supine and standing conditions and $D_{2(max)}$ was found to be significantly different for all regression approaches while $ApEn_{max}$ only for subject-specific using parabolic regression. Linear and parabolic regression formulas were selected to be suitable for the three indices under simulation conditions, although coefficients of determination were slightly lower for $ApEn_{max}$ and *SampEn* than for $D_{2(max)}$.

On the other hand, all nonlinear HRV indices were found still significantly different when corrected by interpolation. It was found a statistically significant reduction in standing with

respect to supine position of 18, 30, and 12% for $D_{2(max)}$, *SampEn,* and $ApEn_{max}$ respectively, mostly reflecting ANS modulation changes while mean HR effect was attenuated. HR-corrected nonlinear index ranges, calculated as the difference of median values for supine and standing positions, were found reduced when compared to uncorrected nonlinear HRV index ranges.

Although, regression formulas were studied as HR-correction approach, their suitability depends on simulation requirements. Possible mismatches of simulated data with respect to real data difficult their application and therefore, we propose to compute nonlinear indices over interpolated RR series to attenuate mean HR effect, since no simulation is required, it saves computational time and still differentiates between both positions. This correction may lead to better understanding complexity and regularity under ANS changes unbiased by mean HR as natural sampling rate of RR time series.

Note that, although HR-correction attenuates the effect of mean HR as sampling rate, HR-corrected nonlinear HRV indices may be still correlated with mean HR due to their physiological dependence. After HR-correction, nonlinear HRV indices are capable of capturing information about ANS modulation in response to body position changes.

HR-corrected nonlinear HRV indices addressed in this study, pointed out a reduction in the complexity of the underlying system and an increase in the HRV series regularity caused by an increase of the sympathetic activity, when changing from supine to standing position, being in agreement with previous works with similar conditions, considering tilt table test or even exercise (Osaka et al., 1993; Kamen et al., 2000; Radhakrishna et al., 2000; Javorka et al., 2002; Porta et al., 2007; Bolea et al., 2014b). Nevertheless, these results and their physiological interpretation are limited by the low number of subjects of study and further studies are needed.

CONCLUSION

In this work, changes in nonlinear HRV indices were studied under different sympathetic conditions where mean HR also changed. It is studied to what extend changes in nonlinear HRV indices are explained by HR ones. Correlation dimension, approximate and sample entropy dependence on mean HR as sampling rate is explored. A simulation study was carried out emulating ANS modulation no linked to mean HR. Simulation results showed that heart rate affects nonlinear indices as it is the intrinsic sampling rate of HRV even when considering the same data length. Two HR-correction methodologies, regression formulas and interpolation, were proposed. Their evaluation on a BPC database revealed a reduction of all studied HR-corrected nonlinear HRV indices in supine and standing positions. After HR-correction, nonlinear HRV indices are capturing changes in the sympathetic modulation by body position-induced changes. HR-correction by interpolation was found suitable to attenuate nonlinear HRV indices effect on mean HR and its application could represent an improvement in their applicability extending it in such cases of non-steady mean HR.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the conception of the work, revising it critically for important intellectual content, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. EP and RB were supervisors of the research and MO gave methodological support. In addition, JB was responsible for drafting the work.

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APPENDIX

Non-linear Method Calculations

Let *x(k), k = 1,...,N* be the time series of interest, *N* being the total number of samples. A set of *m*-dimensional vectors, $y_m(i)$, called reconstructed vectors, can be generated:

$$\mathbf{y}_i^m = \left[x(i), x(i+1), x(i+2), \ldots, x(i+(m-1))\right]^T \quad \text{(A1)}$$

Then, the amount of reconstructed vectors is $N_m = N - (m-1)$ for each *m*-embedded dimension. The distance between each pair of reconstructed vectors, $\mathbf{y}_{\boldsymbol{i}}^{\boldsymbol{m}}$, $\mathbf{y}_{\boldsymbol{j}}^{\boldsymbol{m}}$ is denoted as $d_{i,j}^m$. The norm used is L_∞. Then, each distance is compared with a threshold, *r*, in order to compute how many reconstructed vectors are within a hyper-space centered in the reconstructed vector of reference. The embedded dimension was set to $m = 2$ in this manuscript for the three following methods. Let see it mathematically on each particular case.

Approximate Entropy (*ApEn*)

$$C_i^m(r) = \frac{1}{N_m} \sum_{j=1}^{N_m} H\left(r - d_{i,j}^m\right) \quad \text{(A2)}$$

is the correlation sum where *H* is the Heaviside function.

$$H(x) = \begin{Bmatrix} 1 & x \geq 0 \\ 0 & x < 0 \end{Bmatrix} \quad \text{(A3)}$$

$$\phi^m(r) = \frac{1}{N_m} \sum_{i=1}^{N_m} \log\left(C_i^m(r)\right) \quad \text{(A4)}$$

$$ApEn\,(N, m, r) = \phi^m(r) - \phi^{m+1}(r) \quad \text{(A5)}$$

In addition, due to the intrinsic characteristics of *ApEn*, there is a threshold, *r*, for each time series at which *ApEn* is maximum defining $ApEn_{max}$. Computation of $ApEn_{max}$ is explained in the last subsection of this Appendix.

Sample Entropy (*SampEn*)

Self-comparisons, $d_{i,i}^m$, bias *ApEn* estimation being more notable when data length becomes shorter. Thus, *SampEn* was introduced to solve this bias and to be data length independent. In this case,

$$C_i^m(r) = \frac{1}{N_m - 1} \sum_{j=1, j\neq i}^{N_m - 1} H\left(r - d_{i,j}^m\right) \quad \text{(A6)}$$

is the correlation sum not considering self-comparisons.

$$\phi^m(r) = \frac{1}{N_m} \sum_{i=1}^{N_m} C_i^m(r) \quad \text{(A7)}$$

$$SampEn\,(N, m, r) = -\mathrm{In}\left(\phi^m(r)/\phi^{m+1}(r)\right) \quad \text{(A8)}$$

SampEn is also commonly calculated at the same range of *r* as for *ApEn*, spanning from 0.1 to 0.2 times standard deviation of the data.

Correlation Dimension (*D2*)

$$C_m(r) = \frac{1}{N_m\,(N_m - 1)} \sum_{i,j=1}^{N_m} H\left(r - d_{i,j}^m\right) \quad \text{(A9)}$$

For deterministic systems, $C_m(r)$ decreases monotonically to 0 as *r* approaches 0, and it is expected that $C_m(r)$ is well approximated by $C_m(r) \approx r^{D_2^m}$. For correlation dimension estimation the threshold, *r*, is varied from $r = 0.01$–3 in steps of 0.01.

Thus, D_2^m can be defined as:

$$D_2^m = \lim_{r\to 0} \frac{\log C_m(r)}{\log(r)} \quad \text{(A10)}$$

For increasing *m*, D_2^m values tend to saturate to a value D_2 which constitutes the final correlation dimension estimate. Embedded dimension is varied from $m = 1$–16. In Bolea et al. (2014a), a fast computation algorithm was presented and it is further detailed elsewhere. The algorithm considers self-comparisons in the correlation sums allowing *ApEn* computation in a middle-step. Therefore, $ApEn_{max}$ is computed at the same time as correlation dimension

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Corrigendum: Influence of Heart Rate in Non-linear HRV Indices as a Sampling Rate Effect Evaluated on Supine and Standing

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Comparison of Baroreflex Sensitivity and Cardiac Autonomic Function Between Adolescent Athlete and Non-athlete Boys – A Cross-Sectional Study

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Introduction: It is well known that regular physical activity improves cardiovascular health, and higher baroreflex sensitivity and heart rate variability are associated with cardiovascular health. Adolescence is the age when an individual's behavior is easily modified; early intervention at this stage in terms of physical conditioning or training prevents future cardiovascular risk. Hence, we conceived the present study to assess and compare the baroreflex sensitivity and autonomic function between adolescent athletes and non-athletes.

Methods: We recruited school going athletes (n = 30) and non-athlete boys (n = 30) in the 10–19 age group after obtaining their assent and consent from their parents. We assessed height, weight, heart rate, blood pressure, baroreflex sensitivity, and cardiac autonomic function. Comparison between groups was made using the unpaired t-test for height, weight, body mass index, heart rate, blood pressure, and baroreflex sensitivity and using Mann-Whitney U test for cardiac autonomic function parameters.

Results: There was a trend for higher baroreflex sensitivity in athletes. Heart rate variability (total power and SDNN) was higher in athletes. The parasympathetic tone was higher in terms of higher RMSSD, and higher HF power. Parasympathetic reactivity was higher in athletes in terms of higher 30:15 ratio and EI ratio.

Conclusion: Athletic level physical conditioning has a positive influence on baroreflex function and autonomic function that may prove beneficial to the adolescents' cardiovascular health.

Keywords: heart rate variability, school going, children, baroreflex sensitivity, 30:15 ratio, EI ratio, physical activity

INTRODUCTION

Sedentary lifestyle increases the incidence and prevalence of chronic diseases, especially cardiovascular diseases. WHO predicted that, by 2020, cardiovascular disease will be the leading cause of death. The prevalence of cardiovascular diseases in adolescents is higher in developing countries. Physical activity not only improves physical fitness but also reduces cardiometabolic diseases (Hu et al., 2001). Herein, it is crucial to assess cardiovascular risk among sedentary adolescents, and the effect of physical activity in this age group, which can address the root cause for escalating cardiovascular risk among adolescents.

Baroreceptor reflex mechanism plays a vital role in the short-term regulation of arterial pressure, by the chronotropic effect on the heart and by reflex regulation of parasympathetic and sympathetic outflow to bring about homeostasis. Reduced baroreflex sensitivity (BRS) is associated with hypertension (Mussalo et al., 2002), obesity, diabetes, and metabolic syndrome (Skrapari et al., 2006) in adults. Similarly, in adolescents, reduced BRS is associated with obesity (Lazarova et al., 2009), essential hypertension (Krontoradova et al., 2008), white-coat hypertension (Honzikova and Fiser, 2009), and insulin resistance (Honzikova and Zavodna, 2016). Honzíková et al. (2006) observed reduced BRS even in children and suggested that reduced BRS could be an etiology for hypertension rather than the result of it. Lantelme et al. (2002) showed that BRS correlates well with cardiometabolic risk factors such as age, SBP, DBP, pulse pressure, serum cholesterol, LDL cholesterol, serum triglycerides, and blood glucose. BRS integrates the consequences of these risk factors at various levels and hence might be a comprehensive cardiovascular risk factor (Lantelme et al., 2002) that could be used to predict future cardiovascular events.

Oscillations in the heart rate can be evaluated by heart rate variability (HRV), which is a non-invasive tool to assess the sympathetic and vagal influence on the heart. Available evidence suggests that assessment of BRS and heart rate variability reflects the cardiovascular risk of an individual (La Rovere et al., 2013). Decreased heart rate variability in adolescents is associated with obesity (Guizar et al., 2005), hypertension (Gui-Ling et al., 2013), psychiatric disorders (Blom et al., 2010), diabetes (Wawryk et al., 1997), and developmental changes (Seifert et al., 2014). Documenting the BRS and cardiac autonomic function in adolescents would help us in quantifying their future cardiovascular risk. Further, comparing BRS and cardiac autonomic function between athletes and non-athletes would help us to study the effect of physical activity on these parameters. Lifestyle modifications such as weight reduction (Alvarez et al., 2005) and physical activity (Loimaala et al., 2003) are reported to improve BRS values and cardiac autonomic function (Poirier et al., 2003; Nagai and Moritani, 2004). Adolescence is the age when an individual's behavior can be easily modified, and early intervention at this stage in terms of physical conditioning or training is vital to prevent future cardiovascular risk. Hence, the present study was conceived with the intent to assess and compare the BRS and cardiac autonomic battery of tests between adolescent athletes and non-athletes.

MATERIALS AND METHODS

Study Design

This was a cross-sectional study conducted in Department of Physiology, JIPMER, Puducherry, India, in collaboration with CBSE board schools in Puducherry. The study commenced after obtaining approval from the Institute Ethics Committee for Human Studies (No. JIP/IEC/2013/3/177).

Participants

We considered boys aged 10–19 years, studying in CBSE schools in Pondicherry for the study. Students with a history of cardiovascular, respiratory, or organic disorder which prevents subjects from doing maximal exercise, or on any drugs that could affect vascular and/or autonomic functions or suffering from any acute illness were excluded from the study. We obtained informed written consent from the guardians/parents and written assent from the boys who had met the inclusion criteria. We recruited 30 boys representing their school at state, national, or international level aerobic sports and have undergone athletic level physical conditioning for at least 1 year (Group 1), and 30 age-matched non-athlete students (students participating in recreational sports activities but not in any inter-school athletic events for at least 1 year) were recruited as controls (Group 2).

Parameters Measured

All the participants were requested to report to Autonomic function testing lab, Department of Physiology, JIPMER, Pondicherry, between 8 am and 11 am. We requested the participants to abstain from doing strenuous exercise, and taking caffeinated/alcoholic beverages 12 h before the recording and advised them to have good sleep the previous day of recording. The lab temperature was maintained at ±24°C and dimly lit. Participants were oriented toward the lab, and recording parameters were explained. Demonstration and practice sessions were given for all the procedures before starting the actual recording to alleviate their anxiety. Participants with poor sleep quality, flu, or minor ailments were requested to report on a later date to the recording. On the day of recording, participants were requested to report with an empty bladder, loose clothing, and we instructed them to have their breakfast 2 h before testing.

Anthropometric Parameters

Anthropometric parameters were measured by the International Society for the Advancement of Kinanthropometry (ISAK)-certified investigator. A wall-mounted stadiometer (VM Electronics Hardware Ltd) was used to measure the height accurate to the nearest 0.1 cm. Weight was measured using a digital weighing scale (Charder Electronic Co. Ltd. Taichung, Taiwan, 2013) accurate to the nearest 0.1 kg. Body mass index (BMI) was calculated using the Quetelet index – weight (kg)/height^2 (m^2).

Baseline Cardiovascular Parameters

Blood pressure (BP) and heart rate (HR) were measured after 10 min of rest in the sitting position. Heart rate was measured

manually from the right radial artery. The BP (mm Hg) was recorded from the right arm using mercury sphygmomanometer (Diamond, Industrial-Electronic & allied products, Maharashtra, India). Recordings were taken thrice with 2-min rest intervals, and the average was taken as the final reading. All measurements were taken by the same investigator.

Baroreflex Sensitivity

We measured spontaneous BRS/resting BRS (spontaneous BP changes and HR changes that occur due to respiration) noninvasively by recording pulse rate and finger pressure using Finometer PRO (Finapres Medical Systems BV, Amsterdam, The Netherlands). Following 10 min of supine rest, the finger arterial pressure cuff and brachial artery cuff were placed in the middle finger and 2 cm above the elbow, respectively. Finger arterial pressure waveform was continuously monitored using volume clamp method of Penaz and the Physiocal criteria of Wesseling. Height correction is done using Height correction unit to remove hydrostatic errors caused by the small changes in finger position. Brachial artery pressure (BAP) waveform and level were reconstructed (reBAP) from finger pressure waveform using generalized waveform inverse modeling (Gizdulich et al., 1996, 1997).

Further, individual Riva-Rocci arm cuff return-to-flow pressure level calibration was done and added to reBAP to reduce the inaccuracies in representing the brachial artery pressure (Bos et al., 1996). Pulse rate and interbeat interval (IBI) were calculated from the finger pressure waveform. Personal computer-based data acquisition system Beatscope® Easy (Finapres Medical Systems BV, Amsterdam, The Netherlands) was used to view the data and to calculate BRS. BRS was obtained using time domain cross-correlation method. In this method, heartbeats are spline interpolated on a time scale having 1 s. The cross-correlation function is computed over a sliding correlation window of 12 beats with minimal overlap of five beats at various delays (0–5 s) between blood pressure and pulse interval. Then, the delay with the highest correlation is selected and when its coefficient of determination (r^2) is significant ($p < 0.05$), it is accepted as BRS estimate (Westerhof et al., 2004).

Short-Term Heart Rate Variability

Short-term heart rate variability (HRV) was recorded following Taskforce on Heart Rate Variability-Standards of Measurement, Physiological Interpretation, and Clinical Use. (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996). Subject's skin over the trunk was prepared using abrasive skin preparation gel Nuprep (ELprep, BIOPAC Systems, Inc., CA 93117, USA) for electrode placement. Then disposable EL503 snap electrodes for electrocardiography (ECG) (BIOPAC Systems, Inc., CA 93117, USA) were placed. Electrodes were connected to single-channel ECG100C – electrocardiogram amplifier module (BIOPAC Systems, Inc., CA 93117, USA) which in turn is connected to BIOPAC MP150A-CE Data acquisition unit through Universal interface module UIM100C (BIOPAC Systems, Inc., CA 93117, USA).

After 5 min of supine rest, lead II electrocardiography (ECG) was recorded for 5 min at a sampling rate of 1,000 Hz. The data are visualized using AcqKnowledge® software version 4.1 (BIOPAC Systems, Inc., CA 93117, USA) in Windows-based PC. Ectopics and artifacts were removed manually from the recorded ECG, and RR intervals of 5-min epoch from the lead II ECG recorded are taken in text format. From the RR tachogram, HRV analysis was done using Kubios version 1.0 (Bio-signal analysis Group, Finland). The frequency domain analysis and time domain analysis were computed using Fast Fourier Transformation (FFT) and RR trend, respectively. The frequency domain indices computed were very low frequency (VLF; 0.003–0.04 Hz), low frequency (LF; 0.04–0.15 Hz), and high frequency (HF; 0.15–0.4 Hz), both in absolute powers given as ms^2 and in normalized unit (nu) [LFnu = LF/(TP − VLF) × 100 and HFnu = HF/(TP − VLF) × 100 or HFnu = 1 − LFnu], Total power (TP) ms^2 (TP = VLF + LF + HF) and the LF/HF ratio. The time domain measures include standard deviation of all NN intervals (SDNN), the sum of the squares of differences between adjacent RR intervals (RMSSD), adjacent RR interval differing by more than 50 ms (NN50) and its percentage (pNN50).

Respiration was recorded as a separate channel through the same polygraph with appropriate recommended settings. Respiratory transducer belt (TSD201, BIOPAC Systems, Inc., CA 93117, USA) was placed over the abdomen and connected to respiratory amplifier module (RSP100C, BIOPAC Systems, Inc., CA 93117, USA) and connected to universal interface module UIM100C. Abnormalities concerning the frequency of breathing were carefully analyzed during and after the recording. Any respiratory tracing showing a frequency of more than 24 breaths per min, i.e., 0.4 Hz, was destined to be removed. Exceptional data, if any, were specifically examined and a decision is made regarding the inclusion of the HRV of that subject. However, in this study, such exceptional data were not encountered.

Autonomic Reactivity Tests

Lead II ECG was continuously monitored and recorded when necessary during the following procedures.

Forced Timed Breathing

We asked the subjects to perform forced timed breathing (FTB) at six breaths per minute, comprising inspiratory and expiratory cycles for 5 s each in the supine position (Novak, 2011). We instructed the subjects to avoid sharp inhalation/exhalation/holding of breath during the procedure. Deep breathing was synchronized to a paced voice metronome and, if necessary, guided by hand movement by the investigator. The ratio between maximal RR interval during expiration (E) and minimal RR interval during inspiration (I) is taken as E:I ratio.

Orthostatic Stress Test

Participants were asked to stand within 3 s from their supine position (Low, 2004). The ratio of the longest RR interval around the 30th beat and shortest around 15th beat

(30:15 ratio) was calculated to obtain heart rate response to orthostatic stress test (OST).

Isometric Handgrip Test

Participants were made to sit comfortably in the couch. Maximal voluntary contraction during sustained isometric handgrip by the boys was measured using handgrip dynamometer (Inco, Ambala, India). Isometric handgrip (IHG) test was evaluated at 30% of their maximal strength for 3-min duration. We measured BP during the maneuver in the contralateral arm. The vasoconstrictor response was calculated by calculating the difference between baseline diastolic blood pressure (DBP) and DBP at the end of 2 min during the maneuver (Low, 2004).

Statistical Analysis

Data were subjected to a normality test. Height, weight, BMI, HR, BP, and BRS values passed normality test and are expressed as mean ± standard deviation (SD) and compared using unpaired Student's *t*-test. Short-term HRV parameters and autonomic reactivity test parameters did not pass the normality test and are expressed as median [interquartile range (IQR)] and compared using the Mann-Whitney U test. p <0.05 was considered to be statistically significant.

RESULTS

The anthropometric data including height (cm) (athletes – 159.26 ± 5.66, non-athletes – 160.55 ± 6.21), weight (kg) (athletes – 48.33 ± 5.09, non-athletes – 47.81 ± 5.06) and BMI (kg/m^2) (athletes – 19.05 ± 1.83 and non-athletes – 18.51 ± 1.19) were comparable.

Groups were comparable based on HR (beats per minute) (athletes – 71.20 ± 2.44, non-athletes – 72.50 ± 3.45), systolic blood pressure (mm Hg) (athletes – 108.83 ± 5.12, non-athletes – 109.60 ± 4.75) and diastolic blood pressure (mm Hg) (athletes – 87.67 ± 5.74, non-athletes – 87.83 ± 5.68).

Figure 1 shows that BRS is higher in athletes. However, the difference is not statistically significant.

Table 1 shows a comparison of time domain parameters between the groups. SDNN which denotes overall HRV is statistically higher in athletes. RMSSD (statistically significant), NN50, and pNN50, which denote high-frequency variations (parasympathetic activity), are higher in athletes.

Table 2 shows a comparison of frequency domain parameters between the groups. Total power is significantly higher in athletes, and absolute values of powers in all frequency ranges are significantly higher in athletes; this denotes that short-term heart rate variability is higher in athletes. Based on the normalized units (HFnu and LFnu), relative parasympathetic tone is higher and relative sympathetic tone is lower.

Table 3 shows comparison of autonomic reactivity tests. Orthostatic stress 30:15 ratio is predominantly parasympathetic dependent, while E:I ratio is purely parasympathetic dependent and diastolic rise during isometric handgrip test denotes sympathetic reactivity. 30:15 ratio and E:I ratio are significantly higher in athletes, while diastolic rise during isometric handgrip test is comparable between the groups. Hence, parasympathetic reactivity is higher in athletes while sympathetic reactivity is comparable between the groups.

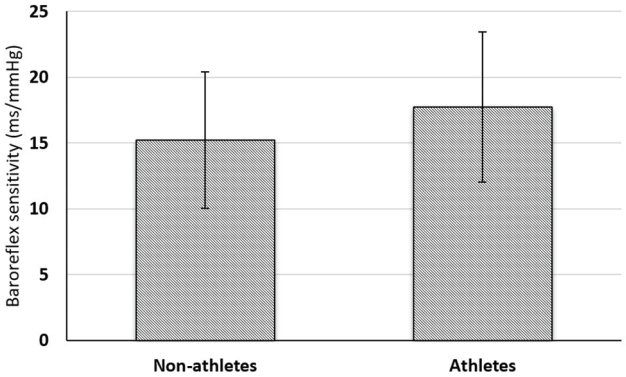

FIGURE 1 | Comparison of baroreflex sensitivity between non-athlete (n = 30) and athlete boys (n = 30). Comparison of BRS (ms/mm Hg) between the groups was done using unpaired Student's *t*-test. *p < 0.05 is considered statistically significant.

TABLE 1 | Short-term heart rate variability – time domain parameters.

Parameters	Non-athlete (n = 30)	Athlete (n = 30)	p
SDNN (ms)	67.20 (97.80)	88.40 (113.30)	0.006
RMSSD (ms)	80.85 (162.50)	108.05 (153.20)	0.034
NN50 (count)	122.50 (192.00)	142.50 (216.00)	0.119
pNN50 (%)	41.75 (55.90)	42.95 (70.40)	0.204

Data presented as median (interquartile range). SDNN, standard deviation of all NN intervals; RMSSD, square root of mean of the sum of the squares of differences between adjacent NN intervals; NN50, number of pairs of adjacent NN intervals differing by more than 50 ms in entire recording; pNN50, number of adjacent NN intervals which differ by more than 50 ms. Comparison between the groups was done using Mann-Whitney U test. p < 0.05 is considered statistically significant.

TABLE 2 | Short-term heart rate variability – frequency domain parameters.

Parameters	Non-athlete (n = 30)	Athlete (n = 30)	p
VLF (ms^2)	128.50 (1219.00)	159.00 (3310.00)	0.301
LF (ms^2)	1475.50 (7635.00)	1839.50 (3681.00)	0.037
HF (ms^2)	1613.50 (8855.00)	3372.00 (8141.00)	0.012
Total power (ms^2)	3354.50 (12561.00)	6130.00 (10167.00)	0.006
LF/HF ratio	0.866 (3.84)	0.631 (1.86)	0.030
LF (nu)	46.42 (59.52)	38.69 (54.85)	0.032
HF (nu)	53.57 (59.52)	61.30 (54.85)	0.032

Data presented as median (interquartile range). VLF, very low frequency (0.003–0.04 Hz); Total power, the variance of NN intervals over the temporal segment; LF, power in low-frequency range (0.04–0.15 Hz); HF, power in high-frequency range (0.15–0.4 Hz); LF (nu), LF power in normalized units [LF/(TP – VLF) × 100]; HF (nu), HF power in normalized units [HF/(TP – VLF) × 100]; LF/HF ratio, ratio LF (ms^2)/HF (ms^2). Comparison between the groups was done using Mann-Whitney U test. p < 0.05 is considered statistically significant.

TABLE 3 | Comparison of autonomic reactivity tests between the groups.

Parameters	Non-athlete (n = 30)	Athlete (n = 30)	p
Orthostatic stress (30:15 ratio)	1.42 (0.68)	1.51 (0.69)	0.025
Forced timed breathing (EI ratio)	1.30 (0.36)	1.40 (0.60)	0.043
Isometric handgrip test	17.00 (2.00)	18 (2.00)	0.293

Data presented as median (interquartile range). 30:15 ratio, the ratio of the longest RR interval recorded after standing to the shortest RR interval recorded during supine position; EI ratio, the ratio of longest RR interval during expiration to shortest RR interval during inspiration; Isometric hand grip test, difference in baseline diastolic blood pressure and diastolic blood pressure after an isometric exercise. Comparison between the groups was done using the Mann-Whitney U test. $p < 0.05$ is considered statistically significant.

DISCUSSION

In the present study, we have assessed BRS and autonomic function in adolescent athlete and non-athlete boys. Age, obesity, and gender are the major factors known to influence BRS (Monahan, 2007; Skrapari et al., 2007; Adoor et al., 2018) and autonomic function (Sharma et al., 2015). To minimize the effects of these factors, we have recruited only age-matched male participants. Further, both groups were comparable based on BMI. Students are from the same locality, socioeconomic background, and studying in CBSE schools. Hence, we believe that the difference between groups due to environmental influence on autonomic function would have been minimal.

Baroreflex sensitivity is measured in terms of change in the interbeat interval (IBI) in milliseconds per unit change in blood pressure in mm Hg. The increase or decrease in IBI in response to decrease or increase in blood pressure by baroreflex might be through any one limb (sympathetic or parasympathetic) of the autonomic nervous system or both together. However, the rapid change is feasible mainly through the parasympathetic limb. Baroreflex sensitivity reflects the complex interaction between the vascular and autonomic function to manage the blood pressure fluctuations of daily life within normal levels. In our study, we observed a trend toward higher BRS in the athlete group. This finding is supported by previous exercise intervention studies done in healthy adults (Iwasaki et al., 2003) and healthy seniors (Okazaki et al., 2005). Higher BRS might be due to the effect of exercise on enhancing the distending capacity of blood vessels and signal transduction in baroreceptors (Monahan et al., 2000; Tanaka et al., 2000a,b) or by improved integration at central cardiovascular centers (Iwasaki et al., 2003).

Reduced BRS is shown to be associated with high blood pressure (Chung and Bruehl, 2008) and resetting of baroreceptor working range to a higher level is seen in hypertension (Krieger, 1988). In our study, even though the BRS is lower in the non-athlete group, the systolic or diastolic blood pressure was comparable with that of the athlete group. We hypothesize that in our study, the level of decrease in BRS is not enough to bring a change in blood pressure or the relation between reduced BRS, high blood pressure, and physical activity observed in previous studies might be due to other unknown mechanisms. In support to our argument, Dutoit et al. have stated that there is no correlation between cardiac baroreflex sensitivity and sympathetic baroreflex sensitivity within healthy young humans particularly in males and BRS measured may not reflect the buffering capacity of the baroreceptor mechanism (Dutoit et al., 2010). Short-term blood pressure regulation is by a neural mechanism such as baroreceptor reflex, and long-term control is by renal mechanisms. The decrease in BRS might manifest only during a demand such as an exercise in early stages and requires some more years or an additional defect in renal mechanism to get reflected in resting blood pressure. It has been shown by Meredith et al. that the effect of physical activity is mainly on renal sympathetic and not on cardiac sympathetic activity (Meredith et al., 1991). However, many authors believe that the reduction in blood pressure is mainly due to a reduction in sympathetic vasomotor tone (Brown et al., 2002; Laterza et al., 2007; Fu et al., 2010).

The relation between cardiac sympathetic overactivity and its association with cardiovascular diseases such as hypertension and heart failure are well established. Sedentary individuals are shown to have higher sympathetic tone even at rest and higher reactivity to any stress (Monda et al., 2011). However, in our study, we observed only a relative increase in sympathetic activity (LFnu and LF/HF ratio) in the non-athlete group. Based on recent publications, the correlation between LF power and sympathetic nerve activity is not clear (Houle and Billman, 1999; Billman, 2013) and, hence, we would not be able to comment about the sympathetic activity in our study based on LFnu and LF/HF ratio alone. Further, LF power was significantly more in athletes than non-athletes. Studies have shown that selective parasympathectomy or cholinergic antagonists reduce LF power by 50% (Akselrod et al., 1981; Randall et al., 1991). Hence, we could hypothesize that the increase in LF power observed in athletes could be due to the parasympathetic component of ANS rather than by sympathetic component. We also did not observe any significant difference in diastolic blood pressure changes to isometric handgrip test between the groups. A decrease in sympathetic activity is expected in physically active individuals as repeated activation of the sympathetic system during each session of exercise is shown to attenuate its response (Grassi et al., 2000; Fraga et al., 2007; Fu et al., 2010). Physical activity is shown to reduced sympathetic activity by reducing weight in overweight or obese individuals (Joyner and Green, 2009) or by increasing muscle mass (Kraus and Levine, 2007) and thereby reducing insulin-mediated sympathetic activity (Julius et al., 1991; Baron et al., 1993). Since the BMI in our study is comparable between the groups, the effect of physical activity might have been minimal in the sympathetic limb of the ANS.

Further, Rosenwinkel et al. have stated that the effect of exercise training on heart rate is mainly due to parasympathetic activity and the sympathetic activity has only minimal impact (Rosenwinkel et al., 2001). This is supported by our findings

from other cardiac autonomic function tests; we observed higher total power and SDNN, which denotes overall increase in heart rate variability; we also observed higher HF power and RMSSD in athletes, which are indicative of resting parasympathetic tone and higher 30:15 ratio and E:I ratio in athletes, which indicates higher parasympathetic reactivity. This goes hand in hand with available evidence that has shown that physical activity enhances parasympathetic activity. Hence, the improved BRS might be due to change in parasympathetic tone or parasympathetic reactivity predominantly. However, in contrast, Bronwyn et al. have argued that exercise training mainly acts on the sympathetic limb of the ANS to improve BRS and it even reduces the parasympathetic contribution on BRS (Kingwell et al., 1992). In our study, we were able to bring out the parasympathetic effect of physical activity, and we were not able to comment on the sympathetic component.

CONCLUSION

Regular physical activity might be beneficial to improve the autonomic tone, which can further improve cardiovascular health as evidenced by higher vagal modulation among athletes than age-matched non-athletes.

LIMITATION

Lack of significant difference in baroreflex sensitivity and other parameters might be due to small sample size. Further, the non-athlete group is not a sedentary population; they were also physically active as part of school co-curriculum. Athlete group consisted of students with various levels of training and competitiveness; this could have influenced BRS and HRV values. We have not quantified the level of physical activity intensity, and its influence on HRV was not studied. The parameters that could have influenced the autonomic activity such as diet, academic stress, and environmental influence were not measured.

FUTURE PERSPECTIVES

A direct measurement such as that of muscle sympathetic nerve activity would help us in determining the effect of physical activity on the sympathetic nervous system.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SS, VS, VA, RR, and AG conceived and designed the analysis. SS, VS, RR, and AG collected the data and contributed data or analysis tools. SS, VS, and RR performed the analysis and wrote the paper.

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Altered Heart Rate Regulation in Adolescent Girls and the Vulnerability for Internalizing Disorders

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Background: The association between decreased heart rate variability (HRV) and increased internalizing symptoms is well documented. Adolescence is a critical period for the development of mental health problems, in particular internalizing symptoms. Previous research has illustrated sex differences in adolescent HRV, such that females have reduced short-term resting state HRV compared to males. Studies on long-term ecological recordings of HRV in adolescents are scarce. The aims of the present study were, (a) to test if adolescent females show decreased long-term HRV and cardiac complexity (CC) compared to males, and (b) to explore whether sex and HRV and CC measures, as well as their interaction, would predict internalizing symptoms.

Materials and Methods: HRV was recorded in $n = 166$ adolescents (86 girls), on a normal school day. HRV and CC measures were calculated on the interbeat interval time series.

Results: Females showed lower HRV and CC in most of the assessed indices. Internalizing symptoms were mainly predicted by HRV whereas sex only predicted symptoms of social anxiety. The interaction between sex and HRV did not predict internalizing symptoms.

Conclusions: Results suggest that reduced HRV should be considered as a potential contributor to exacerbating internalizing symptoms in adolescence. Girls with reduced HRV and CC might be prone to the development of internalizing disorders. HRV is a promising tool for the early identification of vulnerability.

Keywords: heart rate variability, adolescence, sex, depression, anxiety

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INTRODUCTION

Studies have shown that prevalence rates for internalizing disorders (IDs; i.e., depression and anxiety) are higher among females than males (McLean et al., 2011; Beesdo-Baum and Knappe, 2012; Goldman, 2012). Many social and biological factors are discussed to contribute to this discrepancy (Barlow, 2000; Yap et al., 2014). The present study addresses the Negative Valence Systems Domain of the Research Domain Criteria (RDoC) framework (Insel et al., 2010; Insel, 2014) at the physiological autonomic level of analysis, and focuses on sex differences in heart regulation variability and complexity.

Heart rate variability (HRV) refers to the variations in the length of successive interbeat intervals (IBIs), though it is not a unitary concept. By measuring the length of successive intervals, HRV is calculated, and it can be quantified using time domain measures (e.g., the standard deviation of successive intervals or SDNN). However, these measures do not give information about the sources of variability; instead, frequency domain measures of the HRV are used. Thus, use of the high frequency (HF, 0.15–0.40 Hz) band power as an index of vagally mediated HRV is widely accepted, i.e., the variability that depends on the inhibitory action of the autonomic nervous system's parasympathetic branch. The low frequency (LF, 0.4–0.15 Hz) band power has traditionally been interpreted as an index of sympathetic cardiac control. However, recent studies challenge this interpretation and suggest that the LF seems to be a mix of sympathetic, parasympathetic, and other factors (Billman, 2013). Apart from the associations between reduced HF power and IDs (Kemp et al., 2010; Chalmers et al., 2014), a plethora of research has provided support for theoretical models relating reduced vagally mediated HRV and poor emotional regulation (Porges, 1995; Thayer and Lane, 2000, 2009), which is clearly related to many IDs (Aldao et al., 2010). Furthermore, Beauchaine and Thayer (2015) discussed the role of HF HRV as a psychopathological transdiagnostic biomarker within the dimensional framework of the RDoC.

Interest in HRV and cardiac complexity (CC) has grown in the last 30 years. Goldberger (1996) provided a rationale for the need to consider chaos theory and closely related concepts (e.g., fractals) to better understand how physiological systems work, specifically, heart dynamics. Briefly, Goldberger pointed out that classic homeostatic models were unable to explain complex fluctuations in heartbeat. The need to consider the cardiovascular system as a complex system, and specifically the utility of non-linear cardiac measures, has been recently stressed from a clinical perspective by Captur et al. (2017). For a general review on nonlinear, complex biomarkers for emotional disorders (including IDs), see de la Torre-Luque et al. (2016). According to the findings of this meta-analysis, the cardiovascular systems of healthy people show a higher level of CC than the systems of those who suffer from an ID.

The median age of onset for many IDs is during adolescence (Kovacs and Devlin, 1998; Kessler et al., 2005). A recent meta-analysis (Koenig et al., 2017) has shown, through short-term recordings, that adolescent females display lower vagal activity and a higher mean heart rate (HR) than adolescent males under resting conditions. Several studies have also correlated reduced HRV with internalizing symptoms. Dietrich et al. (2007) found that higher HR and lower respiratory sinus arrhythmia (the influence of respiration on HR and another index of the vagally mediated HRV) were related to increased internalizing symptoms, even in healthy adolescents, and Greaves-Lord et al. (2010) found that low respiratory sinus arrhythmia predicted anxiety levels in healthy adolescent females 2 years later, but not in males. Few studies have looked at sex differences using longer cardiac recordings. Faulkner et al. (2003) studied sex differences in HRV measurements taken from 24-h ECG recordings in early adolescents ($n = 43$, mean age = 15), and found lower SDNN values for females, but no differences in HF power. Silvetti et al. (2001) used several time domain measures of HRV and found higher SDNN in males in 24-h recordings from 103 children and adolescents (1–20 years old), though no sex differences were found in other measures (e.g., RMSSD, root mean squares of successive IBI differences). Bobkowski et al. (2017) did not find sex differences in HRV or in CC measures derived from Poincaré plots calculated from 24-h recordings from 100 children (aged 3-18 years old). Our previous study (Fiol-Veny et al., 2018) examined sex- and anxiety-related differences in HRV and CC on adolescents with high- versus low- anxiety scores (n = 95, mean age = 14) through long cardiac ecological recordings. There was no interaction between sex and anxiety, but adolescents with high anxiety symptoms showed lower HRV than their low-anxious counterparts, whereas females showed lower HRV and lower CC than males. However, we underlined that future research should also investigate sex-related differences regardless of the level of anxiety, i.e., assessing boys and girls that not only encompass high and low-anxiety students, but all ranges of anxiety symptom levels.

The wide age range of the samples in some of these studies and the methodological differences (e.g., length and conditions of the ECG recordings or the specific measure of HR regulation) could explain the incongruence of results, although most of them point out that cardiac regulation is less flexible in adolescent girls compared to boys. Importantly, HRV and CC in regular school settings (instead of more conventional lab settings) have rarely been studied, though this ecological assessment could provide helpful knowledge about heart regulation in everyday conditions (see Bornas et al., 2015).

Therefore, the present study aimed to: (a) to test if adolescent females show decreased HRV and CC compared to males and (b) to explore whether sex and measures of HRV and CC, as well as their interaction, predict levels of internalizing symptoms.

MATERIALS AND METHODS

The Bioethics Committee at the university approved of all procedures, and all participants and their parents or legal guardians provided written consent.

Participant Selection

Data for the present analyses were taken from a sizable longitudinal project (TRANS; Bornas et al., 2014), the general aim of which was to examine the trajectories of anxiety symptoms and physiological correlates of anxiety in adolescents over the course of 3 years. Self-reported symptoms of anxiety were assessed by repeated measurements every 6 months. All participants were middle-class Caucasians from both urban and rural areas on the island of Majorca, Spain. Unlike our previous study from the TRANS project (Fiol-Veny et al., 2018), participants' selection encompassed not only those with extremely high or low scores of anxiety symptoms. The full range of scores was covered in the present study to overcome this limitation, and a much larger and non-overlapping sample was used. According to Quintana (2017), a sample size of $n = 82$ per group is required to achieve a statistical power of 90% to detect

effect sizes in HRV case-control studies. In order to achieve this sample size, and considering the average number of students per school enrolled in the TRANS project, five of the 12 schools were randomly selected and invited to participate in the present study. All of them agreed, and therefore data from 169 adolescents were collected. The exclusion criteria were: suffering from severe mental retardation; a neurological, developmental, or psychiatric disorder (American Psychiatric Association, 2000); or any severe cardiovascular or respiratory disease, as per medical records that were reported by parents. As we examined HRV and CC in a sample of healthy adolescents, students with any ID diagnoses were excluded ($n = 3$). The final sample ($N = 166$) comprised $n = 86$ girls and $n = 80$ boys.

Procedure

First, we administered an online version of the self-report questionnaire (see instruments below) to all participants. In the following month we conducted cardiac recordings on a regular school day within the academic context but only when participants were not going to be doing any physical or out-of-the-ordinary activities during the time of the recording. Participants left the classroom in the early morning (around 8:00 a.m.) and went to a private room to have their BMIs calculated via measurements of height and weight. Subsequently, the researcher put two electrodes on each participant's chest and attached a portable device to the electrodes (see the Cardiac Assessment section). Participants then returned to their classrooms with the devices activated, and cardiovascular function was continuously measured for the first 120 min of that day. Finally, each participant was scheduled for a structured interview with one of the researchers. Adolescents who had consumed alcohol, drugs, and/or caffeinated beverages during the previous 4 h, adolescents with an acute illness, or those who were menstruating were assessed another day.

Instruments

Psychological Assessment

We used the Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000) to assess anxiety and depression symptoms. It is a 47-item, self-report questionnaire, with sub-scales assessing separation anxiety disorder, social anxiety, generalized anxiety disorder, panic disorder, obsessive compulsive disorder, and major depressive disorder. There is an overall scale indicating the total level of anxiety symptomatology. The RCADS requires respondents to rate how often each item applies to them. Items are scored 0–3, with 0, 1, 2, and 3, respectively corresponding to "never," "sometimes," "often," and "always." The internal consistency of the subscales within the present sample ranged from $\alpha = 0.80$ to $\alpha = 0.94$.

The Mini-International Neuropsychiatric Interview for Children and Adolescents (M.I.N.I. Kid; Sheehan et al., 1998) was used to determine if an ID was present. This is a structured diagnostic interview for children from 6 to 17 years of age based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (American Psychiatric Association, 2000) and the International Statistical Classification of Diseases, 10th Revision (World Health Organization, 1992).

Participants filled out other questionnaires required by the TRANS study: The Early Adolescence Temperament Questionnaire (EATQ-Revised long form; Ellis and Rothbart, 2001) and the Sensitivity to Punishment and Sensitivity to Reward Questionnaire Junior version (SPSRQ-J; Torrubia et al., 2008).

Cardiac Assessment

The reporting of cardiac measures follows GRAPH guidelines (Quintana et al., 2016). The Firstbeat Bodyguard 2© (Firstbeat Technologies Ltd., Jyväskylä, Finland) is a measurement device that is attached to the skin with two electrodes: one on the left side of the chest and the other on the right side under the collarbone. The device continuously records beat-to-beat HR (i.e., normal to normal intervals or IBIs) with a sampling frequency of 1,000 Hz; the recordings lasted 120 min. The first and last 15 min of each time series were removed to eliminate the adaptation period and the period when participants stopped their regular activity. Thus, each time series analyzed was 90 min long, and the number of IBIs in each time series depended on the participant's HR. We visually inspected each recording to exclude distorted signals due to apparatus failure ($n = 2$ out of $n = 168$, 1.19%). The time series was filtered with the Physionet (Goldberger et al., 2000) HRV toolkit (http://www.physionet.org/tutorials/hrv-toolkit/) a low-pass band filter at 1,100 ms, a high-pass band filter at 400 ms, and a central interval filter were applied. Using a window of 11 intervals (5 intervals on either side of the central interval) but excluding the central interval, the average over the window was calculated. If the central interval laid outside 20% (0.20) of the window average, this interval was excluded and the window was advanced to the next interval, resulting in the mean of excluded IBIs ($n = 229$ out of $n = 11808$, 1.94%).

HRV calculation

Time domain measures were based on the beat-to-beat (or normal to normal, NN) intervals. We used the AVNN (average of all NN intervals), a measure of HR, and RMSSD.

Frequency domain measures provided the total spectral power of all NN intervals in each frequency band by transforming the time series into the frequency domain through the fast Fourier transform. LF ranges from 0.04 to 0.15 Hz and HF from 0.15 to 0.40 Hz.

All HRV measures were calculated using the Physionet (Goldberger et al., 2000) HRV toolkit.

CC calculation

The detrended fluctuation analysis (DFA) is a method designed by Peng et al. (1995), which allows for the estimate of temporal power-law form correlations embedded in IBI time series. We obtained two scaling exponents: the α1 exponent, which reflects short-term scaling (4-11 beats), and the α2 exponent, which reflects long-term scaling (>11 beats). $\alpha 1 > \alpha 2$ in healthy individuals. For uncorrelated data $\alpha = 0.5$, while $0.5 < \alpha < 1$ indicates persistent long-range power-law correlations. Both exponents were calculated using Kubios HRV Software, version 2.1 (Tarvainen et al., 2014).

For the fractal dimension (FD), the allometric aggregation method examines the invariance of the relationship between the mean and the standard deviation of a data series as the data points are iteratively aggregated, thus decreasing the resolution (scale) of the series. First, the mean and standard deviation of the time series of length N (x_1, x_2, x_3,..., x_n) are calculated. Secondly, each pair of adjacent points is aggregated ($x_1 + x_2$, $x_3 + x_4$,..., $x_{n-1} + x_n$) to get a time series of length $N/2$, and the mean and standard deviation of that time series are calculated. We repeate this aggregation process for 3, 4, 5, and usually up to $N/10$ adjacent data points. Since our IBI time series included around 9000 values, we repeated the aggregation process for 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 adjacent data points and then plotted the mean and standard deviation of each level of aggregation on a logarithmic graph. When the number of aggregated values increases, the relationship between mean and standard deviation remains the same (invariant) and can therefore be described as a linear relationship, with the slope of the line being the scaling exponent h. Notice that the scaling exponent for random fluctuations is 0.50 and for deterministically regular processes it is 1 (in both cases the time series would not be self-similar or fractal). If we take the variance instead of the standard deviation, the slope of the line is $2h$. A MathWorks Matlab code (available upon request) was developed to calculate the exponent. The fractal dimension is then calculated according to the formula FD = 2-h.

The multiscale entropy analysis (Costa et al., 2002) evaluates the entropy (or irregularity) of complex time series at different time scales. It creates consecutive coarse-grained time series by averaging a successively increasing number of data points in non-overlapping windows. Then, sample entropy (SampEn; Richman and Moorman, 2000), which is the negative natural logarithm of the conditional probability that two sequences with similar m points remain similar at the next point, is calculated for each of the coarse-grained time series. Less entropy implies higher regularity and predictability. In the current study, entropy for scale factors 1, 5, 10, 15, and 20 was calculated using the software available from https://physionet.org/physiotools/mse/.

Analytical Strategy

According to recommendations made by Quintana (2017) regarding the sample size required to detect medium effect sizes in HRV studies with a statistical power > 80%, around 80 participants of both sexes should be allocated to each group. We explored differences in age and BMI between males and females, through between-group t-tests, and differences in internalizing symptoms using one-way MANOVAs. The alpha level was adjusted for multiple comparisons (Bonferroni) and set to 0.007. Since HF power values were not normally distributed (Shapiro-Wilk's $W = 0.885$, $p < 0.001$, skewness = 1.49, kurtosis = 3.10), we took the natural logarithm of these values (lnHF) before conducting statistical analyses that require data to be normally distributed. After this transformation, the lnHF distribution was normal ($W = 0.98$, $p > 0.05$, skewness = −0.47, and kurtosis = 0.31). Cardiac measures were adjusted by the average HR to control for HR-derived mathematical bias. The cardiac measures that correlated negatively with HR were divided by the average HR. Those which correlated positively (only DFA 1) were multiplied by the average HR, in line with Gasior et al. (2016) and Sacha et al., 2013a,b. All subsequent statistical analyses were conducted with both adjusted and unadjusted cardiac measures.

We analyzed differences in cardiac measures between males and females using one-way MANOVAs. Then, Cohen's d effect size values were calculated, and they were interpreted according to Quintana (2017). The research team grouped the cardiac measures into four sets of MANOVAs, and alpha levels were corrected according to Bonferroni: with time domain measures in the first set ($p < 0.025$), frequency-domain measures in the second set ($p < 0.025$), DFA and FD in the third set ($p < 0.017$), and entropy measures in the fourth set ($p < 0.01$). Multiple linear regressions analyses were also performed with each internalizing symptoms subscale as the dependent variable, and both sex and cardiac measures as predictors. In step 1, sex (coded 1 for boys, and 2 for girls) and each cardiac measure were entered as predictors. In step 2, the interaction between sex and each cardiac measure was added. To prevent for multicollinearity, values of cardiac measures were standardized. All analyses were conducted using the IBM SPSS Statistics v.20.0.0 package.

RESULTS

The sample in the present study was comprised of two groups: 86 girls (mean age $M = 14.96$, $SD = 0.46$, range = 13.78–16.40 years old; mean body mass index [BMI] = 17.68, $SD = 2.74$) and 80 boys (mean age $M = 14.96$, $SD = 0.36$, range = 14.42–16.26 years old; mean BMI = 18.19, $SD = 3.35$).

No significant differences in age or BMI were found between males and females. Sex differences in cardiac measures are shown in **Table 1**. Females showed higher mean HR and decreased variability in both time-domain and frequency-domain measures when compared to males ($p < 0.025$, according to the Bonferroni correction). Females also showed lower CC than males ($p < 0.017$ for the FD, and $p < 0.010$ for sample entropy measured at scale factors 15 and 20). The effect sizes can be considered medium as most of them were close to 0.50. When we analyzed adjusted cardiac indices, similar results were obtained but the differences between males and females increased. Furthermore, differences also became significant for the α2 exponent ($p = 0.002$, $d = 0.49$) and entropy at scale factors 5 ($p = 0.006$, $d = 0.44$) and 10 ($p = 0.002$, $d = 0.50$).

Means, standard deviations, and ranges of total internalizing symptoms are shown in **Table 2** and separated by sex. Sex differences in internalizing symptoms are also presented in **Table 2**. No significant differences were found between males' and females' internalizing symptoms ($p > 0.007$).

Hierachical multiple regression analyses predicting internalizing symptoms from sex and unadjusted cardiac measures are shown in **Table 3** (HRV measures) and **Table 4** (CC measures). For unadjusted cardiac data, sex was a significant predictor, but of social anxiety only. HR predicted social anxiety, panic, obsessive-compulsive, depression, and total anxiety symptoms. LnHF predicted social anxiety, depression and total anxiety symptoms. Depression was also predicted by the

α1 exponent entropy at scale factors 1 and 5. We also found interaction effects between sex and the α1 exponent in the prediction of depression symptoms. The interaction between sex and entropy at scale factors 5 and 10 also predicted separation anxiety symptoms.

Scatter plots of HR and lnHF (both unadjusted by HR) with social anxiety, depressive and total anxiety symptoms are shown in **Figure 1**.

When we adjusted cardiac indices by HR, sex was a significant predictor of social anxiety symptoms (β ranged from 0.163 to 0.195, $p < 0.05$). HR predicted social anxiety ($\beta = 0.161$, $p = 0.044$), panic ($\beta = 0.202$, $p = 0.013$), obsessive-compulsive symptoms ($\beta = 0.287$, $p = 0.000$), depression ($\beta = 0.199$, $p = 0.014$), and total anxiety ($\beta = 0.212$, $p = 0.009$). LnHF predicted social anxiety ($\beta = -0.169$, $p = 0.036$), panic ($\beta = -0.183$, $p = 0.026$), obsessive-compulsive symptoms ($\beta = -0.217$, $p = 0.008$), depression ($\beta = -0.182$, $p = 0.026$), and total anxiety ($\beta = -0.190$, $p = 0.020$). The α1 exponent was a significant predictor for social anxiety ($\beta = 0.186$, $p = 0.020$), panic ($\beta = 0.235$, $p = 0.004$), obsessive-compulsive symptoms ($\beta = 0.269$, $p = 0.001$), depression ($\beta = 0.233$, $p = 0.004$), and total anxiety ($\beta = 0.232$, $p = 0.004$). The α2 exponent predicted panic ($\beta = -0.166$, $p = 0.045$), obsessive-compulsive symptoms ($\beta = -0.238$, $p = 0.004$), and total anxiety ($\beta = -0.171$, $p = 0.038$). The FD was a significant predictor for panic ($\beta = -0.177$, $p = 0.031$), obsessive-compulsive symptoms ($\beta = -0.246$, $p = 0.002$), depression ($\beta = -0.173$, $p = 0.033$), and total anxiety ($\beta = -0.184$, $p = 0.024$). Entropy at all scale factors predicted obsessive-compulsive (β from −0.241 to −0.204, $p < 0.05$) and total anxiety (β from −0.202 to −0.163, $p < 0.05$) symptoms. Social anxiety symptoms were predicted by entropy at scale factors from 5 to 20 (β from −0.178 to −0.160, $p < 0.05$). Separation anxiety symptoms were predicted by entropy at scale factor 5 ($\beta = -0.168$, $p = 0.041$). Panic symptoms were predicted by entropy at scale factors 1 and 5 ($\beta = -0.177$, $p = 0.029$ and $\beta = -0.185$, $p = 0.024$, respectively). Finally, depression symptoms were predicted by entropy at scale factors 1, 5, and 20 ($\beta = -0.158$, $p = 0.050$; $\beta = -0.202$, $p = 0.013$; and $\beta = -0.175$, $p = 0.035$, respectively). No interaction effects of sex and cardiac measures were found.

TABLE 1 | Mean, standard deviation (in parenthesis) and comparison of cardiac measures (unadjusted by HR) between males and females.

	Males ($n = 80$)	Females ($n = 86$)	$F_{(1, 164)}$	p	Cohen's d
HR	89.33 (11.006)	93.421 (10.407)	6.11	0.014	0.39
RMSSD	38.16 (13.89)	33.24 (12.31)	5.93	0.016	0.38
LF	1913.67 (767.09)	1614.65 (686.96)	7.10	0.008	0.42
lnHF	6.26 (0.69)	5.96 (0.76)	6.71	0.010	0.41
DFAα1	1.40 (0.14)	1.42 (0.13)	1.37	0.243	0.18
DFAα2	0.98 (0.08)	0.96 (0.07)	4.30	0.040	0.32
FD	1.10 (0.03)	1.08 (0.02)	11.60	0.001	0.53
MSE sf1	0.90 (0.23)	0.87 (0.19)	0.87	0.351	0.15
MSE sf5	1.51 (0.19)	1.47 (0.17)	2.96	0.087	0.27
MSE sf10	1.53 (0.13)	1.49 (0.13)	3.63	0.058	0.30
MSE sf15	1.51 (0.12)	1.45 (0.12)	9.06	0.003	0.47
MSE sf20	1.49 (0.12)	1.43 (0.12)	10.14	0.002	0.50

HR, Heart rate (beats per minute); RMSSD, square root of the mean of the squares of differences between adjacent NN intervals (ms); LF, low frequency power (ms^2); lnHF, logarithmic transformation of high frequency power; DFA α1, alpha 1 exponent calculated by Detrended Fluctuation Analyses; DFA α2, alpha 2 exponent calculated by Detrended Fluctuation Analyses; FD, Fractal Dimension; MSE sf1, multiscale entropy scale factor 1; MSE sf5, multiscale entropy scale factor 5; MSE sf10, multiscale entropy scale factor 10; MSE sf15, multiscale entropy scale factor 15; MSE sf20, multiscale entropy scale factor 20.

DISCUSSION

Reduced HRV is associated with IDs (Kemp et al., 2010; Chalmers et al., 2014). The present study aimed to examine sex differences in HR regulation in middle adolescence, a typical time of ID onset (Kovacs and Devlin, 1998; Kessler et al., 2005). We followed a flexible, non-categorical approach to study the ID spectrum (following RDoC framework and under the Negative Valence Systems Domain), considering the physiological unit of analysis. Since the development of these disorders takes places in our daily lives, we decided to record cardiac activity during participants' routine school activities.

Sex differences were found in most of the HRV measures that support the main hypotheses. Females showed higher and less variable HR in the time domain, i.e., the length of successive IBIs did not change as much as in males. When we looked at the spectral components of HR, we found decreased vagal activity (lower HF power) in females. Low vagally-mediated HRV

TABLE 2 | Mean, standard deviation and range of total internalizing symptoms, and separated between males and females.

	Total			Males ($n = 71$)			Females ($n = 83$)					
Variable	***M***	***SD***	**Range**	***M***	***SD***	**Range**	***M***	***SD***	**Range**	***F* (1, 152)**	***p***	**Cohen's *d***
Social anxiety	8.85	5.53	0–25	7.62	4.86	0–25	9.92	5.88	0–25	6.83	0.010	0.43
Generalized anxiety	5.82	3.61	0–15	5.69	3.61	0–15	5.94	3.63	0–15	0.18	0.670	0.07
Separation anxiety	1.56	2.49	0–16	1.62	2.79	0–16	1.51	2.22	0–10	0.08	0.928	0.05
Depression	8.04	5.64	0–23	7.25	5.23	0–23	8.71	5.92	0–23	2.58	0.110	0.26
Obsessive compulsive	3.11	3.35	0–16	3.07	3.25	0–16	3.14	3.45	0–15	0.02	0.892	0.02
Panic	4.71	4.85	0–21	4.67	4.94	0–21	4.75	4.79	0–19	0.01	0.928	0.02
Total anxiety	24.06	16.21	0–83	22.67	15.79	0–83	25.25	16.55	0–76	0.97	0.327	0.16

TABLE 3 | Hierachical multiple regression analyses predicting internalizing symptoms from sex and HRV measures (unadjusted by HR).

	Internalizing symptomatology													
	Social A		GAD		Separation A		PD		OCD		Depression		Total anxiety	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.068**		0.004		0.025		0.040*		0.081**		0.055*		0.050*	
Sex		0.183*		0.026		−0.046		−0.023		−0.032		0.099		0.048
HR		0.161*		0.056		0.157		0.202*		0.287**		0.199*		0.212**
Step 2	0.001		0.000		0.001		0.002		0.000		0.001		0.000	
Sex × HR		0.092		0.068		−0.090		−0.127		0.006		−0.097		−0.004
Step 1	0.066**		0.007		0.008		0.016		0.020		0.037		0.028	
Sex		0.184*		0.023		−0.036		−0.012		−0.011		0.107		0.057
RMSSD		−0.155		−0.076		−0.085		−0.127		−0.144		−0.144		−0.151
Step 2	0.016		0.020		0.002		0.004		0.015		0.016		0.016	
Sex × RMSSD		−0.394		−0.437		−0.136		−0.203		−0.388		−0.392		−0.394
Step 1	0.061**		0.005		0.002		0.001		0.006		0.021		0.014	
Sex		0.186*		0.025		−0.028		0.004		−0.002		0.118		0.066
LF		−0.136		−0.059		−0.032		−0.023		−0.081		−0.068		−0.088
Step 2	0.003		0.010		0.000		0.000		0.004		0.000		0.003	
Sex × LF		−0.163		−0.316		0.048		−0.037		−0.197		−0.020		−0.170
Step 1	0.069**		0.011		0.009		0.021		0.024		0.046*		0.034	
Sex		0.182*		0.020		−0.037		−0.015		−0.013		0.103		0.054
lnHF		−0.165*		−0.098		−0.093		−0.147		−0.156		−0.173*		−0.168*
Step 2	0.010		0.008		0.000		0.004		0.012		0.010		0.009	
Sex × lnHF		−0.331		−0.302		−0.006		−0.205		−0.364		−0.333		−0.318

N = 154. HR, heart rate (beats per minute); RMSSD, square root of the mean of the squares of differences between adjacent NN intervals; LF, low frequency power; lnHF, logarithmic transformation of high frequency power; Social A, social anxiety symptoms; GAD, generalized anxiety symptoms; Separation A, separation anxiety symptoms; PD, panic symptoms; OCD, obsessive compulsive symptoms.
$^*p < 0.05$; $^{**}p < 0.01$.

has been repeatedly reported to be associated with anxiety (see Chalmers et al., 2014; Bornas et al., 2015) and depression (Kemp et al., 2010). In our study, females showed lower LF power. The meaning of the differences in LF is far from clear (Billman, 2013), but our results show that the vagal influence on the HR in everyday life at school is lower in adolescent females compared to males.

Previous studies using 24-h ECG recordings have provided inconclusive results. Bobkowski et al. (2017) did not find sex differences in HRV or in CC, whereas Faulkner et al. (2003) and Silvetti et al. (2001) reported higher SDNN in males than in females. However, we must note that the ages of the samples in these studies are hardly comparable: Faulkner et al. (2003) studied 75 adolescents, aged 15 years old (SD = 1.6 years); Silvetti et al. (2001) included 103 participants from 1 to 20 years old; and Bobkowski et al. (2017) studied 100 children and adolescents aged 3-18. The age group in this latter study cannot be properly compared to Faulkner's study sample. The age range of the current study sample was much closer to Faulkner's, and the results confirm the same findings using a much larger sample size, i.e., females show reduced HRV when compared to males. The meta-analysis conducted by Koenig et al. (2017) is similar in that females displayed lower vagally-mediated HRV in short recordings under resting conditions. However, a direct comparison of these studies and the current study should be made with caution due to the different conditions in which the cardiac recordings were taken. A more proper comparison could be done with our previous study (Fiol-Veny et al., 2018), which was also based on 90 min long ecological recordings. It was found that adolescent females (mean age = 14) showed lower HRV and CC than males. However, we took an a priori groups-based approach, selecting adolescents above the 75th percentile or below the 25th percentile in anxiety symptomatology. The current study overcomes this limitation, considering all ranges of anxiety symptomatology (and also depression symptoms), and using a non-overlapping and larger sample size. Importantly, the previously reported differences between males and females are confirmed by the current results.

Considering the complex regulation of HR, we found the DFA α2 exponent to be higher (closer to 1) in males than in females, but only when adjusted by HR. DFA quantifies the fractal-like properties of IBI time series data. The α2 exponent gives a measure of these properties for long-range (>11 beats) correlations, and Peng et al. (1995) showed that α2 = 1 in healthy people. Though statistically significant, the difference between females and males could be physiologically minimal as both exponents (adjusted by HR) were close to 0.010 (0.011 for males and 0.010 for females, Cohen's d = 0.49).

TABLE 4 | Hierachical multiple regression analyses predicting internalizing symptoms from sex and CC measures (unadjusted by HR).

	Internalizing symptomatology													
	Social A		GAD		Separation A		PD		OCD		Depression		Total anxiety	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.064**		0.007		0.007		0.037		0.020		0.049*		0.034	
Sex		0.196*		0.028		−0.029		−0.008		0.000		0.115		
DFAα1		0.144		0.079		0.081		0.192*		0.140		0.180*		0.166*
Step 2	0.016		0.015		0.002		0.007		0.014		0.034*		0.016	
Sex × DFAα1		0.393		0.387		0.144		0.259		0.379		0.579*		0.398
Step 1	0.045*		0.003		0.001		0.000		0.002		0.022		0.007	
Sex		0.200*		0.041		−0.019		0.006		0.004		0.141		0.077
DFAα2		−0.050		0.043		0.023		−0.006		−0.049		0.077		−0.016
Step 2	0.004		0.000		0.017		0.003		0.001		0.002	−0.132		
Sex × DFAα2		−0.196		−0.069		−0.411		−0.162		−0.120		0.005		−0.219
Step 1	0.073**		0.007		0.004		0.003		0.007		0.030		0.020	
Sex		0.188*		0.031		−0.037		−0.005		−0.001		0.123		0.064
FD		−0.154		−0.069		−0.056		−0.056		−0.082		−0.097		−0.110
Step 2	0.001		0.003		0.019		0.001		0.000		0.001		0.000	
Sex × FD		−0.081		−0.169		0.437		−0.104		0.066		−0.102		−0.015
Step 1	0.055*		0.002		0.019		0.030		0.033		0.043*		0.030	
Sex		0.203*		0.033		−0.029		0.000		0.003		0.122		0.073
MSEsf1		−0.110		−0.025		−0.136		−0.172*		−0.182*		−0.163*		−0.153*
Step 2	0.000		0.000		0.010		0.002		0.002		0.000		0.001	
Sex × MSEsf1		−0.030		−0.048		0.316		0.121		0.135		−0.054		0.113
Step 1	0.056*		0.012		0.018		0.015		0.021		0.048*		0.028	
Sex		0.196*		0.024		−0.036		−0.005		−0.004		0.111		0.065
MSE sf5		−0.113		−0.104		−0.135		−0.124		−0.145		−0.177*		−0.150
Step 2	0.001		0.003			0.553*		0.242		0.314		0.139	0.009	
Sex × MSEsf5		0.107		0.169		0.031*		0.006		0.010		0.002		0.296
Step 1	0.049*		0.008		0.007		0.000		0.002		0.019		0.011	
Sex		0.197*		0.023		−0.034		0.008		0.006		0.123		0.071
MSE sf10		−0.081		−0.086		−0.084		0.003		−0.039		−0.047		−0.067
Step 2	0.001		0.002		0.034*		0.010		0.010		0.003		0.010	
Sex × MSEsf10		−0.091		−0.155		0.608*		0.330		0.323		0.192		0.325
Step 1	0.051*		0.010		0.006		0.000		0.001		0.018		0.010	
Sex		0.187*		0.014		−0.039		0.012		0.003		0.121		0.065
MSE sf15		−0.094		−0.094		−0.072		0.022		−0.036		−0.037		−0.065
Step 2	0.000		0.001		0.018		0.002		0.006		0.000		0.003	
Sex × MSEsf15		−0.026		0.106		0.458		0.147		0.260		0.062		0.201
Step 1	0.049*		0.010		0.006		0.000		0.004		0.020		0.011	
Sex		0.189*		0.012		−0.041		0.010		−0.005		0.115		0.063
MSE sf20		−0.077		−0.096		−0.078		0.014		−0.067		−0.063		−0.069
Step 2	0.000		0.000		0.002		0.001		0.003		0.000		0.000	
Sex × MSEsf20		−0.055		0.043		0.163		−0.098		0.182		0.007		0.024

N = 154. DFA α1, alpha 1 exponent calculated by Detrended Fluctuation Analyses; DFA α2, alpha 2 exponent calculated by Detrended Fluctuation Analyses; FD, Fractal Dimension; MSE sf1, multiscale entropy scale factor 1; MSE sf5, multiscale entropy scale factor 5; MSE sf10, multiscale entropy scale factor 10; MSE sf15, multiscale entropy scale factor 15; MSE sf20, multiscale entropy scale factor 20; Social A, social anxiety symptoms; GAD, generalized anxiety symptoms; Separation A, separation anxiety symptoms; PD, panic symptoms; OCD, obsessive compulsive symptoms.

*$p < 0.05$; **$p < 0.01$.

We should look at the results obtained through allometric aggregation to complete this picture. These results revealed significant sex differences (Cohen's $d = 0.46$ for adjusted and Cohen's $d = 0.53$ for unadjusted data) in that the FD of males' HR was higher than the FD of females (and therefore, heart regulation seemed to be more complex in males than in females).

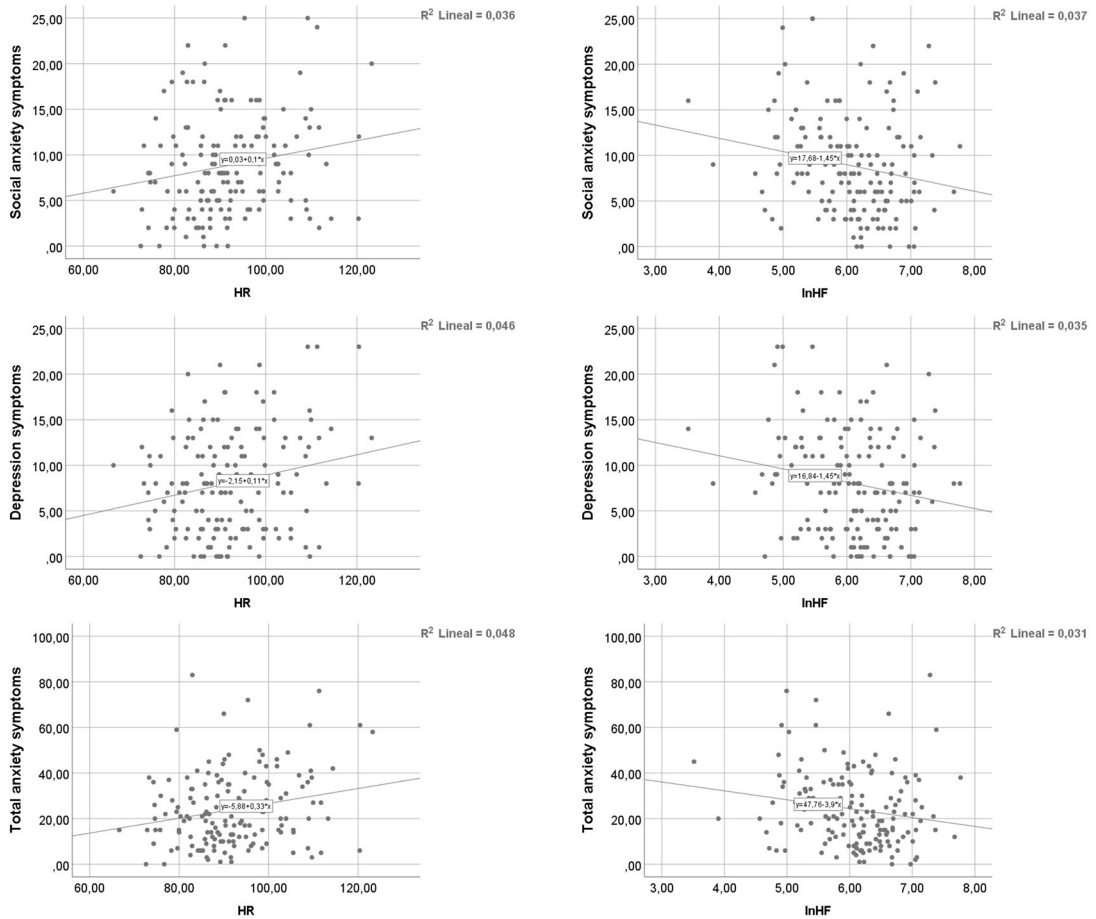

FIGURE 1 | Scatter plots of HR and lnHF with social anxiety **(upper panels)**, depressive **(medium panels)**, and total anxiety **(lower panels)**.

Again, the physiological meaning of the small differences in the absolute mean values prevents a clear interpretation of this finding, but both the α2 exponents and the FD values point to poorer HR regulation in females. Furthermore, sex differences in HR entropy were clear at scale factors 15 and 20 with unadjusted data, and at scale factors from 5 to 20 with adjusted data, with females showing more regular and predictable HRs. By increasing the scale factor, the differences also increased (Cohen's $d = 0.15$ for scale factor 1, and $d = 0.50$ for scale factor 20). Together, these findings clearly underline the more complex regulation of HR in males than in females. We should say that HR scaling properties underlie the role of vagal activity (indexed in this study by the HF band power), as pointed out by Balle et al. (2015). Hence, it is not only the real-time function of the parasympathetic system that is reduced in females, but the HR fractal properties, too.

The second aim of this study was to explore whether sex and measures of HRV and CC, as well as their interaction, predicted levels of internalizing symptoms. We should say that no prediction analysis could be performed in our previous study (Fiol-Veny et al., 2018) because of its methodological design. Therefore, the current study adds important information on the predictive role of HRV and CC on several internalizing symptoms. For unadjusted data, the regression models showed that social anxiety symptoms were predicted by HR and lnHF. Depression symptoms were predicted by HR, lnHF, the α1 exponent, and entropy at scale factors 1 and 5. Panic, obsessive-compulsive, and total anxiety symptoms were also predicted by HR. The regression models for adjusted cardiac indices showed that panic, obsessive-compulsive symptoms, depression, and total anxiety were predicted by HR, lnHF, the α1 exponent, the FD, and entropy at scale factors 1 and 5. Social anxiety symptoms were also predicted by HR, lnHF, the α1 exponent, and entropy from scale factors 5 to 10. The α2 exponent predicted panic, obsessive-compulsive, and total anxiety symptoms. Obsessive-compulsive and total anxiety symptoms were predicted by entropy at scale factors from 10 to 20. Separation anxiety symptoms were predicted by entropy at scale factors 5 and 10. Finally, depression was predicted by entropy at scale factor 20. If we look at the direction of these relationships, we see that the lower HRV or CC is, the higher internalizing symptoms are.

Interestingly, the regression analysis for both adjusted and unadjusted data showed that sex did not predict the self-reported

scores, except in the case of social anxiety. For unadjusted data we found interaction effects between sex and the α1 exponent in the prediction of depression symptoms, and between sex and entropy at scale factors 5 and 10 in the prediction of separation anxiety symptoms. However, when we analyzed the adjusted indices, the interaction between sex and cardiac measures was not significant in any regression model, meaning that the effect of the cardiac measure on the internalizing symptoms did not differ by sex. Thus, even though adolescent females are more "physiologically" vulnerable, they did not feel more depressed or anxious than their male counterparts, except in social conditions. The greater social anxiety experienced by females might be influenced by several factors, such as different levels of pubertal maturation or social demands (for instance, adolescents spend several hours at school, a context where high social demands are common).

We might be able to understand the lack of sex differences in self-reported anxiety and depression from a diathesis-stress model at the onset of anxiety and depressive disorders. According to Christiansen (2015) and Hyde et al. (2008), biological sex differences result in an inherent vulnerability toward anxiety and depression in females, and cultural influences may further increase sex differences in developing specific IDs through the stress component of the diathesis-stress model (for a review, see Belsky and Pluess, 2009). When HRV is below a certain value, the individual might be more prone to developing an ID, but that ID would not be the unavoidable consequence of the physiological condition. For instance, girls may or may not show higher anxiety scores than boys, but they could still be more physiologically vulnerable to developing an ID due to their lower HRV and CC. However, whether they will develop an ID also depends on other factors, such as the contextual conditions in which they live.

To sum up, our results provide some evidence supporting the role of HRV and CC as relevant factors for the early identification of physiological vulnerability of adolescent girls. From a clinical point of view, the fact that girls and boys do not significantly differ in self-reported levels of anxiety or depression may mask the girls' vulnerability, and consequently, it might hinder the development of strategies to protect girls against anxiety.

Although we consider reduced HRV and CC as vulnerability factors for IDs, we are aware that a more global, neurovisceral view (see Thayer and Sternberg, 2006) is necessary to build a theory on physiological vulnerability to IDs. This view includes brain structures (e.g., the amygdala) and allostatic systems (e.g., the hypothalamic-pituitary-adrenal axis) where sex differences exist (Lenroot and Giedd, 2010). However, it is far beyond the scope of this report to undertake such a neurovisceral review.

It is also important to note that studies on healthy adults have reported that females' HRV and CC are usually greater than they are in males (Ryan et al., 1994; Koenig and Thayer, 2016). Since HR regulation changes throughout the lifespan (O'Connor et al., 2008; Moodithaya and Avadhany, 2012), further longitudinal research should focus on when these patterns invert along the course of development and why. Perhaps the higher HRV and CC in some adult females come from an effort to balance out anxiety or depression. In fact, Thayer et al. (1998) reported that females suffering from depression showed more heart period variability compared to their counterparts without depression. More recently, Williams et al. (2015) found that the relationship between 5-min resting HF-HRV and negative psychological states can be positive in women. Although this result could point to an overcompensation of heart regulation as a way to overcome anxiety or depression, it is insufficient in women with internalizing symptoms or disorders.

Concerning the clinical implications of the results of the present study, adolescence could be the right time to use strategies aimed at preventing IDs. However, we cannot base these strategies solely on self-reported anxiety scores since healthy adolescent girls do not show higher scores than their counterparts at that age (except in social anxiety, in our study). What is different, depending on sex, is HR regulation, which is significantly less variable and complex in females, and might be making them more prone to developing IDs. From physiologically based interventions (e.g., HRV biofeedback, see Lehrer and Gevirtz, 2014) to psychological treatments (e.g., cognitive behavioral therapies, see Garanaki et al., 2009), a wide range of procedures are available to set up and implement those preventive strategies in adolescent girls who might be "physiologically" vulnerable to IDs. The results of the current study should be helpful in identifying such girls. The regression models suggest that the lower the HRV or CC, the higher the risk for IDs.

We are aware of the controversy around the need to adjust for HR when measuring HRV. In order to shed light on this issue, we encourage future research to analyze adjusted and unadjusted HRV measures.

The current study has various limitations that should be considered in future studies. First of all, we have not addressed whether sex differences in HR regulation would arise in stressful laboratory conditions. Studies on vagal reactivity are important to better understand the physiological components of complex heart regulation in highly stressful situations, but we wanted to examine how adolescent males and females regulate their HR in common everyday situations, regardless of how stressful they perceive these conditions to be. The results obtained in reactivity studies could be biased since everyone reacts to stress, but differences in HRV and CC in daily life can more reliably reflect "physiological" vulnerability.

Secondly, we did not monitor participants' activities while the cardiac devices were recording. We assumed that these activities (usually listening to teachers or working in the classroom) would be equivalent, though we cannot be sure. Lastly, we did not register the different phases of the menstrual cycle. Although we assessed menstruating participants on another day, it has been suggested that all menstrual stages have an effect on the brain areas related to HR regulation (Bäckström et al., 2011). However, in our opinion, the size of the group of females was large enough to ensure these limitations would not influence the results.

Despite these shortcomings, the present study uses a large sample of adolescents that is homogeneous with regards to the age range studied. Furthermore, the fact that adolescents were

monitored under ecological conditions improves the external validity of the findings. Altogether, it makes the results of our study robust and helpful in comprehending how sex differences in HR regulation could contribute to the onset of internalizing disorders.

AUTHOR CONTRIBUTIONS

AF-V has contributed in the study design, collection, analysis and interpretation of data, and in the writing of the manuscript. AD has contributed to the collection and analysis of data and in writing the manuscript. MB has contributed to the study design and editing the manuscript. XB is the main researcher of the more in-depth project where this study took place. He has contributed to the study design, analysis and interpretation of data, as well as in writing and editing the manuscript.

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Sleep Instabilities Assessed by Cardiopulmonary Coupling Analysis Increase During Childhood and Adolescence

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The electrocardiogram-based cardiopulmonary coupling (CPC) technique may be used to track sleep instabilities. With progressing age, maturational changes during childhood and adolescence affect sleep. The objective was to assess developmental changes in sleep instabilities in a natural setting. ECGs during nighttime sleep on regular school days were recorded from 363 subjects aged 4 to 22 years (204 females). The estimated total sleep time (ETST) decreased from 598 to 445 min during childhood and adolescence. Stable sleep linearly decreased with progressing age (high frequency coupling (HFC): 70–48% ETST). Unstable sleep [low frequency coupling (LFC): 9–19% ETST], sleep fragmentation or disordered breathing (elevated LFC: 4–12% ETST), and wake/REM states [very low frequency coupling (VLFC): 20–32% ETST] linearly increased with age. Hence, with progressing age the sleep of children and adolescents shortens, becomes more unstable and is more often affected by fragmentation or sleep disordered breathing, especially in the age group >13 years. It remains to be clarified whether some of the changes are caused by a social jetlag, i.e., the misalignment of body clock and social time especially in adolescents.

Keywords: cardiopulmonary coupling analysis, childhood and adolescence, sleep electrocardiogram, sleep quality

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INTRODUCTION

Many physiological functions are associated with age-related changes during childhood and adolescence. Sleep is also considerably altered during this period, e.g., sleep duration declines with progressing age. The recommended sleep duration over 24 h for pre-schoolers is 11–12 h whereas teenagers should sleep 9 h on average (Galland et al., 2012; Hirshkowitz et al., 2015; Paruthi et al., 2016). The decline in sleep duration is accompanied by a considerable delay of sleep time. Preschoolers go to bed early in the evening and wake up early in the morning whereas adolescents go to bed late and wake up later in the morning (on free days) (Roenneberg et al., 2004; Foster and Roenneberg, 2008). This change of sleep preferences is accompanied by a progressive delay of the body clock by up to 2 h during childhood and adolescence. At the same time sleep patterns also change: during non-rapid eye movement (non-REM) sleep, e.g., the amount of slow wave sleep (1–4 Hz delta EEG) decreases whereas the amount of stage 2 sleep increases with progressing age

(Scholle et al., 2011; Carskadon and Dement, 2017). At the same time the average amplitude of delta oscillations decreases whereas its mean frequency increases during non-REM sleep (Feinberg and Campbell, 2013). These examples illustrate that sleep is not a static function. Instead, the restorative and regenerative function of sleep depends on age and it obviously adapts to the demands and requirements of the organism at the respective age.

Sleep instabilities during non-REM sleep, as assessed by the occurrence of cyclic alternating patterns (CAP) in the EEG, also show age-related changes during childhood and adolescence (Parrino et al., 2012). The CAP rate, i.e., the percentage ratio of CAP time to non-REM sleep time, is low during infancy and increases with progressing age, shows a peak at puberty and decreases during adolescence. Although the term 'instability' may suggest a negative significance, these sleep instabilities are an essential part of the sleep microstructure because they take part in the formation of sleep cycles. Furthermore, the amount of CAP, i.e., the amount of unstable sleep, correlates to cognitive performance: in normal children at the age of 3–12 years, the CAP rate was positively correlated to the capability of non-verbal fluid reasoning (Bruni et al., 2012; Novelli et al., 2013).

The assessment of sleep instabilities in children so far has been based on the analysis of appearance of CAP in polysomnographic data with a relatively low number of subjects, e.g., 10 children (Bruni et al., 2002, 2005). It has been shown that sleep instabilities also have an impact on cardiac autonomic regulation as assessed by spectral analysis of heart rate variability (HRV) (Ferri et al., 2000; Ferini-Strambi et al., 2000). Hence, we focus on the assessment of stable and unstable sleep by means of an electrocardiogram-based technique called cardiopulmonary coupling (CPC) (Thomas et al., 2005). The analysis of CPC quantifies the degree of coherent coupling between HRV and variations of the R-wave amplitude caused by modulations of the respiratory tidal volume. CPC of high frequency oscillations [high frequency coupling, (HFC)] indicates stable sleep, i.e., non-CAP sleep, because vagal modulations induced by respiration (i.e., respiratory sinus arrhythmia) prevail in the cardiac autonomic regulation. Low frequency coupling (LFC) is associated with sleep instabilities, i.e., CAP sleep, because recurring sympathetic arousals lead to recurring short episodes of increased of heart rate in the low frequency range. A special characteristic of LFC, so-called elevated LFC, can be used to detect periods of apnea and hypopnea (Thomas et al., 2007). Furthermore, the extent of HFC is related to the amount of slow wave oscillations in the EEG (Thomas et al., 2014). In a pediatric population, HFC has also been observed to correlate negatively with sleep-disordered breathing (Guo et al., 2011).

The purpose of this study was to assess changes of sleep instabilities during childhood and adolescence assessed by the CPC technique. This technique is applied to ECG data from a larger cohort that we have previously investigated with respect to changes of cardiac autonomic regulation during childhood and adolescence (Cysarz et al., 2011, 2013). The 24-h ECG data were acquired on regular school days and, hence, we assess maturational changes of ECG derived sleep characteristics in a context that highly shaped daily activities.

MATERIALS AND METHODS

Subjects

ECG recordings from a previous study were re-analyzed (Cysarz et al., 2011). Initially, 469 subjects were enrolled in the cross-sectional study. 363 (age range 4–22 years; 204 females, 159 males) subjects had an ECG recording suitable for the nighttime sleep analysis, i.e., a continuous ECG recording free of major artifacts was available for the entire sleep. The subjects were divided into the following four age groups to allow comparisons among the groups: (1) pre-schooler, age <7 years (mean 5.7 ± 0.9 years; $N = 74$, 47 females); (2) primary school children, $7 \leq$ age <10 years (mean 8.5 ± 0.8 years; $N = 99$, 54 females); (3) early adolescence, $10 \leq$ age < 14 years (mean 11.8 ± 1.1 years; $N = 89$, 48 females); and (4) adolescence, age >14 years (mean 17.8 ± 2.2 years, $N = 101$, 55 females). This age classification is closely related to an age based classification using clinical parameters (Kliegman et al., 2016).

None of the subjects had any history of cardiovascular disease. With respect to sleep disturbances 4 subjects had diseases (e.g., bronchial asthma) that could have affected sleep. Five subjects were taking medication for attention-deficit hyperactivity disorder and four subjects were receiving naturopathic treatment which may have affected sleep. A *post hoc* analysis comparing the results of the entire group and the results of the groups without the above-mentioned subjects showed no differences. Hence, all subjects were included in the analysis.

Ethics Statement

Written informed consent was obtained from the child's guardian and, if applicable, also from the child in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Charité – Universitätsmedizin Berlin.

ECG Recordings

Twenty-four hour-Holter ECGs were recorded under regular school day conditions during the week. The subjects were introduced to aim of the ECG recording and the technical handling of the device. Furthermore, information for parents (or legal guardians) was provided to support the handling. Subsequently, they were equipped with the Holter device at school in the morning and the recording was stopped the next morning. The digital Holter device (Medilog MK3, Schiller-Engineering, Graz, Austria) had a built-in R-peak detection with a precision of <1 ms (internal sampling rate: 4096 Hz). The ECG (at a sampling rate of 256 Hz) as well as the automatically identified times of the R-peaks were transferred to a PC for further analysis. The times of the automatically identified R-peaks were inspected manually using the saved ECG. In case of ventricular extrasystoles, artifacts and premature beats the timings of the related beats were marked accordingly ($<0.5\%$ of all R-peaks). Times of normal beats that were not correctly identified by the device's built-in R-peak detection were corrected manually

on the basis of the saved ECG. Hence, the times of these corrected beats had a precision of 4 ms (<1.5% of all R-peaks).

Cardiopulmonary Coupling Analysis

The nighttime sleep period was part of the 24 h-ECG recordings. Bedtime and wake-up time had to be noted in a diary by the subjects or their legal guardians. In many cases these diary sleeping times were not available. Hence, in order to consistently assess sleep times, the following procedure was used to detect sleep onset and end of sleep. It has been shown that cardiac autonomic regulation clearly changes during the onset of sleep. The average RR-interval, the very low frequency component of HRV and the ratio of the low frequency component to the high frequency component, LF/HF, decrease during the onset of sleep (Trinder et al., 2001; Shinar et al., 2006; Kuo et al., 2008). Using a customized program, two diagrams were displayed one above the other. In one diagram, the series of RR-intervals and its 30 s moving average was plotted versus time (i.e., the occurrences of R-peaks). In a second diagram, the ratio LF/HF was plotted for each successive 30 s interval. A clear increase of RR-intervals (i.e., a drop of heart rate) accompanied by a clear decrease of LF/HF in the evening was defined as the time of onset of sleep. In rare cases only LF/HF showed a clear decrease in the evening whereas the clear increase of the RR-intervals was delayed. In these cases the timing of decrease of LF/HF was evaluated as the onset of sleep. The wake-up time was defined as a clear decrease of RR-intervals in the morning accompanied by a clear increase of LF/HF. This procedure could only estimate the total sleep time and is, hence, termed 'estimated total sleep time' (ETST).

The analysis of CPC relies on cross-spectral and coherency estimates between variations of heart rate and its associated changes of tidal volume, i.e., ECG-derived respiration (EDR). The EDR signal consisted of the series of R-peak amplitudes (Moody et al., 1985, 1986). Two properties need to be fulfilled for CPC: (1) both signals need to oscillate at a given frequency. This is quantified by cross-spectral analysis. (2) The signals need to be coupled and synchronized. This property is assessed by the analysis of coherence, i.e., the phases of both signals must be aligned so that the phase relationship is constant. The product of these two properties is the key in the quantification of the degree of CPC (Thomas et al., 2005).

All calculations were carried out as recently described to assure comparability of the results with findings of previous studies (Thomas et al., 2005). First, outliers in the RR-interval series were removed. The RR-interval series and its EDR signal were re-sampled at 2 Hz using cubic spline interpolation. The cross-spectral power and the coherence of these two signals was calculated of a 1024 sample window (approx. 8.5 min) using the Fourier transformation. For each analysis window the sum of the two maximal coherent cross-power peaks in the very low frequency band (0–0.01 Hz), the low frequency band (0.01–0.1 Hz) and the high frequency band (0.1–0.4 Hz) was used to assess physiological couplings. HFC was associated to respiratory sinus arrhythmia and deep sleep. This is termed stable sleep and is linked to non-CAP sleep. On the other hand, the presence of LFC is associated with CAP sequences, i.e., unstable sleep. Furthermore, wake/REM periods are associated to the appearance of very low frequency coupling (VLFC). Using appropriate thresholds, sleep can be categorized into HFC (stable sleep), LFC (unstable sleep), and VLFC (wake/REM state). We note that the detection thresholds varied across studies dealing with CPC (Thomas et al., 2005, 2009; Ibrahim et al., 2010). Here, we used the thresholds that were established in the initial study (Thomas et al., 2005).

Sleep instabilities caused by sleep disordered breathing are associated with an elevated low frequency coupling (e-LFC), a subset of LFC (Thomas et al., 2007). We also assessed the amount of e-LFC of each ECG recording. VLFC, LFC, HFC, and e-LFC were calculated as ratio of the total duration of each state to the ETST. The percentage ratio of the changes of the sleep states VLFC, LFC, and HFC between successive analysis windows to the total number of analysis windows was termed 'sleep fragmentation.' A lower percentage ration indicates fewer changes of sleep states, i.e., a less fragmented sleep.

In addition, the CAP-rate and the proportion of non-REM sleep were assessed using VLFC, LFC, and HFC. The CAP-rate, i.e., the percentage ratio of the total CAP time to non-REM sleep time, is assessed as the percentage ratio of the LFC time to the combined LFC and HFC time. The proportion of non-REM sleep time (time spent in sleep stages N1–N3) to the total sleep time is estimated by the proportion of the combined HFC and LFC time, i.e., time spent in stable and unstable non-REM sleep, to the ETST. All calculations were carried out using customized Matlab programs.

Statistical Analysis

Each parameter was plotted vs. age to describe the dependency on age. The average time course was plotted by means of a moving average (window length: 21 data points). The dependency of the parameters on age was modeled by a regression analysis using a 3rd order polynomial. The significance of this modeling was calculated using the F-statistic.

The results of the four age groups are presented as mean ± SD. The parameters were normally distributed as indicated by the Kolmogorov–Smirnov test ($p < 0.01$). Comparisons between the groups were calculated using the 1-way ANOVA procedure. The *post hoc* comparisons were Bonferroni corrected. Student's *t*-test was used to compare results of male and female subjects. A value $p < 0.05$ was considered statistically significant.

RESULTS

Generally, all investigated parameters showed age related changes and a linear model was sufficient to describe the changes. The average ETST according to the linear model ($R^2 = 0.35$, $p < 0.001$) was 598 min (10.0 h) for the pre-schoolers and declined to 445 min (7.4 h) at the end of adolescence (see **Figure 1A**). The four age groups reflected this decline (see **Figure 2A**; <7 years: 571 ± 60 min, 7–9 years: 565 ± 47 min, 10–13 years: 539 ± 45 min, >13 years: 477 ± 64 min; $p < 0.001$). The age group >13 years had the shortest ETST compared to

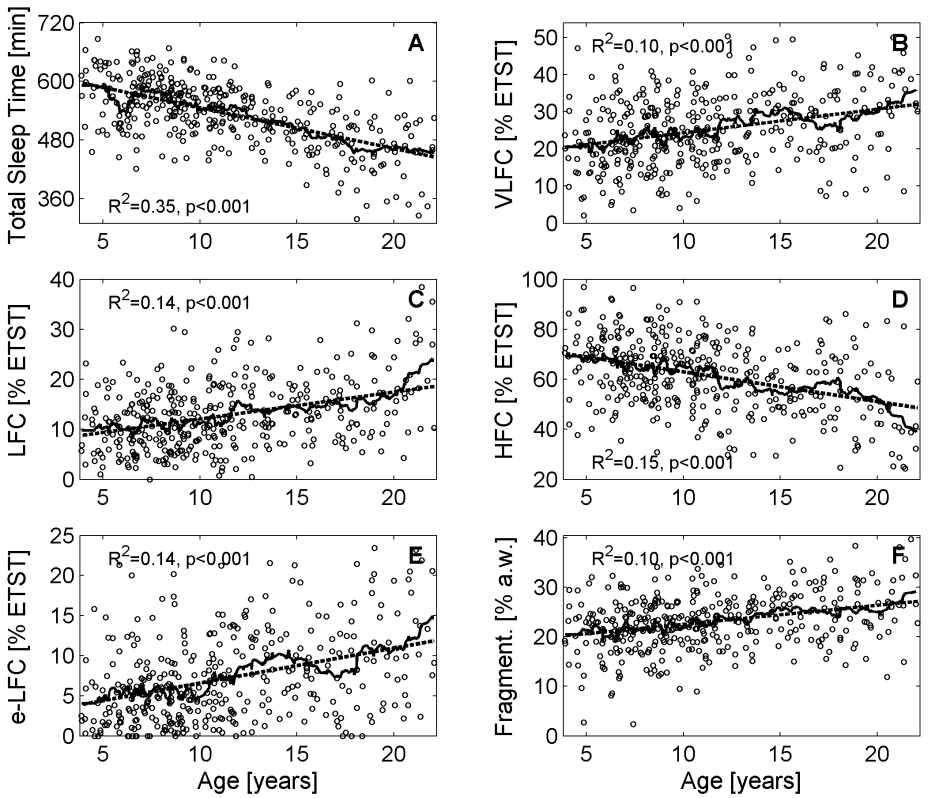

FIGURE 1 | Variations of cardiopulmonary coupling (CPC) parameters during childhood and adolescence. **(A)** Estimated total sleep time (ETST), **(B)** Very low frequency coupling (VLFC) indicating REM/wake states, **(C)** Low frequency coupling (LFC) indicating unstable non-REM sleep, i.e., cyclic alternating pattern (CAP) sleep, **(D)** High frequency coupling (HFC) indicating stable non-REM sleep, i.e., non-CAP sleep, **(E)** Elevated low frequency coupling (e-LFC) indicating disordered breathing, and **(F)** fragmentation of sleep. The solid lines show the moving average (21 consecutive data points) and the dashed line indicates the linear regression model.

the other age groups ($p < 0.05$). Furthermore, also the age group 10–13 years was different to the age group 7–9 years and the age group <7 years ($p < 0.05$).

Very low frequency coupling, i.e., REM/wake periods, was lowest for the pre-schoolers (approx. 20% of the ETST; see **Figure 1B**) and increased to approx. 32% ETST at the end of adolescence (linear model: $R^2 = 0.10$, $p < 0.001$). The age groups also reflected the increase (see **Figure 2B**; <7 years: 21.5% ± 9.6% ETST, 7–9 years: 23.3% ± 8.4% ETST, 10–13 years: 25.6% ± 9.0% ETST, >13 years: 29.3% ± 9.7% ETST; $p < 0.001$). The age group >13 years had the greatest VLFC compared to all other age groups ($p < 0.05$) and also the coupling in the age group 10–13 years was greater than that of the age group <7 years ($p < 0.05$).

Low frequency coupling, i.e., unstable non-REM sleep, increased with progressing age (see **Figure 1C**). The pre-schoolers showed LFC in approx. 9% ETST whereas LFC was approx. 19% ETST at the end of adolescence (linear model: $R^2 = 0.14$, $p < 0.001$). The age groups showed an increase of LFC especially for the group >13 years (see **Figure 2C**; <7 years: 10.0% ± 5.5% ETST, 7–9 years: 11.2% ± 5.6% ETST, 10–13 years: 13.0% ± 6.9% ETST, >13 years: 16.1% ± 7.1% ETST; $p < 0.001$). The age group >13 years had the greatest LFC compared to all other age groups ($p < 0.05$) and also the coupling of the age group 10–13 years was greater than that of the age group <7 years ($p < 0.05$).

High frequency coupling, i.e., stable non-REM sleep, decreased with progressing age from approx. 70% ETST for the pre-schoolers to approx. 48% ETST at the end of adolescence (linear model: $R^2 = 0.15$, $p < 0.001$; see **Figure 1D**). The age groups also showed a decrease (see **Figure 2D**; <7 years: 67.8% ± 13.9% ETST, 7–9 years: 64.9% ± 12.1% ETST, 10–13 years: 60.9% ± 13.8% ETST, >13 years: 53.7% ± 14.7% ETST; $p < 0.001$). The age group >13 years showed a clearly reduced HFC compared to all other age groups ($p < 0.05$). Furthermore, the coupling of the age group 10–13 years was lower compared to that of the age group <7 years ($p < 0.05$).

The estimated proportion of non-REM sleep for the pre-schoolers was approx. 79% of the ETST. At the end of adolescence, the proportion decreased to approx. 67% (linear model: $R^2 = 0.10$, $p < 0.001$). The age groups reflected this decrease: age group <7 years: 77.8% ± 10.0% ETST, age group 7–9 years: 76.1 ± 8.5% ETST, age group 10–13 years: 73.9% ± 9.3%, age group >13 years: 69.8% ± 10.1%. The age group >13 years had the lowest estimated proportion of non-REM sleep compared to all other age groups ($p < 0.05$).

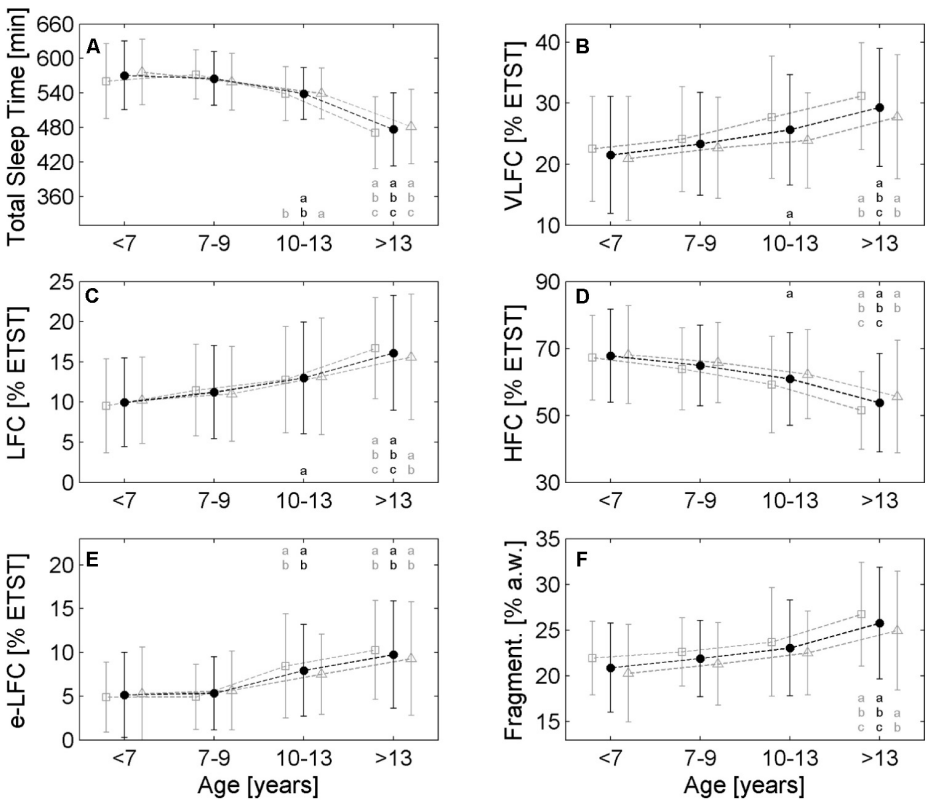

FIGURE 2 | Comparison of different age groups with respect to the cardiopulmonary coupling (CPC) parameters. **(A)** Estimated total sleep time (ETST), **(B)** Very low frequency coupling (VLFC) indicating REM/wake states, **(C)** Low Frequency Coupling (LFC) indicating unstable non-REM sleep, i.e., cyclic alternating pattern (CAP) sleep, **(D)** High Frequency Coupling (HFC) indicating stable non-REM sleep, i.e., non-CAP sleep, **(E)** Elevated low frequency coupling (e-LFC) indicating disordered breathing and **(F)** fragmentation of sleep. Each age group shows three values: the average of the entire group is plotted in the middle (black points), open squares on the left of each black point show the average of male subjects and open triangles show the average of female subjects. The small letters in each diagram refer to significant differences ($p < 0.05$) with respect to the following age group: a: <7 years, b: 7–9 years, c: 10–13 years. Black letters refer to the whole group, gray letters on the left and right of the black letter denote males and females, respectively.

The estimated CAP-rate increased from 11% of total non-REM time for the preschoolers to 29% of total non-REM time at the end of adolescence (linear model: $R^2 = 0.16$, $p < 0.001$). Accordingly, the age groups showed an increase with increasing age: <7 years: 13.6% ± 8.7% est. non-REM time, 7–9 years: 15.2% ± 8.5% est. non-REM time, 10–13 years: 18.5% ± 11.1% est. non-REM time, >13 years: 24.2% ± 12.4% est. non-REM time. The age group >13 years showed a clear increase compared to all other age groups ($p < 0.05$) and also the age group 10–13 years was higher compared to the age group <7 years ($p < 0.05$).

Elevated LFC (e-LFC), i.e., fragmented sleep or sleep disordered breathing, increased with progressing age. e-LFC was lowest for the pre-schoolers (approx. 4% ETST) and increased to approx. 12% ETST at the end of adolescence (linear model: $R^2 = 0.14$, $p < 0.001$; see **Figure 1E**). Accordingly, the age groups also showed an increase of e-LFC (see **Figure 2E**; 5.1% ± 4.9% ETST, 7–9 years: 5.3% ± 4.2% ETST, 10–13 years: 7.9% ± 5.2% ETST, >13 years: 9.7% ± 6.1% ETST; $p < 0.001$). The age group > 13 years and the age group 10–13 years had a higher e-LFC compared to the age groups 7–9 years and <7 years ($p < 0.05$).

The relative number of changes of sleep states between successive analysis windows (a.w.), i.e., sleep fragmentation, also showed an increase with progressing age: 20% changes in relationship to all analysis windows for the pre-schoolers and 27% changes at the end of adolescence (linear model: $R^2 = 0.10$, $p < 0.001$; see **Figure 1F**). The age groups also reflected the increase of the fragmentation (see **Figure 2F**; 20.9% ± 6.0% a.w., 7–9 years: 21.9% ± 5.1% a.w., 10–13 years: 23.1% ± 4.9% a.w., >13 years: 25.8% ± 5.7% a.w.; $p < 0.001$). The age group >13 years had a higher fragmentation compared to all other age groups ($p < 0.05$).

Gender differences were generally weak. The diagrams in **Figure 2** show slightly higher VLFC, LFC, e-LFC and sleep fragmentation in male subjects compared to female subjects especially for the age group >13 years. On the other hand, HFC and ETST are slightly lower in male subjects compared to female subjects. However, only VLFC showed a clear difference in the age group 10–13 years: male subjects had greater VLFC compared to female subjects (27.7% ± 10.0% ETST vs. 23.9% ± 7.8% ETST,

$p < 0.05$). In this age groups the male subjects also tended to a lower proportion of estimated non-REM sleep compared to female subjects (72.0% ± 10.3% ETST vs. 75.5% ± 8.2% ETST, $p = 0.08$). Furthermore, in the age group >13 years the male subjects tended to greater VLFC compared to female subjects (31.2% ± 8.8% ETST vs. 27.8% ± 10.2% ETST, $p = 0.08$).

DISCUSSION

Assessing sleep parameters by means of the CPC technique revealed a clear increase of unstable sleep with progressing age as indicated by LFC and the estimated CAP-rate. At the same time also the amount disordered breathing (assessed by e-LFC), the amount of REM/wake (assessed by VLFC) states and the amount of sleep fragmentation increased. On the other hand, stable sleep as indicated by HFC and also the estimated proportion of non-REM sleep decreased with progressing age. Hence, all parameters consistently indicate a change in sleep quality that occurs most pronounced at the end of adolescence. This decrease is accompanied by a decrease of total sleep time with progressing age.

The average ETST showed a decline of approx. 150 min from infants to the end of adolescence. This decline is in accordance with the time course of total sleep time from polysomnographic recordings (Ohayon et al., 2004; McLaughlin Crabtree and Williams, 2009; Scholle et al., 2011; Carskadon and Dement, 2017). Hence, also the course of other sleep parameters (e.g., sleep period time, sleep efficiency, time spent in sleep stages N1–N3 and REM) should be comparable to previous studies even though we were not able to track them by the ECG based approach.

Generally, wake-up time should be constant throughout childhood and adolescence because the children had to be at school punctually. Hence, the decline in total sleep time with progressing age can only be explained by later bedtimes in the evening. This delay in bedtime is in accordance with the delay of the body clock by up to 2 h with progressing age (Roenneberg et al., 2004). In contrast, it has been shown that the sleep times are considerably longer on free days because the sleep amount is then determined by the body clock rather than the alarm clock, i.e., need to be at school in time (Roenneberg et al., 2012). The difference in sleep time caused by the difference of body or circadian clock and social clock is termed social jet lag and can be as large as 2 h (Wittmann et al., 2006; Roenneberg et al., 2012).

The estimated amount of non-REM sleep for the young children, i.e., pre-schoolers and primary schoolers is in accordance with the portion retrieved from polysomnographic recordings (approx. 80%) (Ohayon et al., 2004; Scholle et al., 2011). However, previous studies with polysomnographic recordings showed a proportion of non-REM sleep of approx. 75–80% also for adolescents (Ohayon et al., 2004; Scholle et al., 2011; Carskadon and Dement, 2017). Hence, the decrease of non-REM sleep time with progressing age estimated by HFC and LFC underestimates the amount of non-REM sleep for adolescents.

Unstable sleep is associated with the occurrence of CAP sequences and is quantified by LFC of CPC analysis. LFC increased during childhood and adolescence suggesting that the amount of CAP sequences during sleep increased during childhood and adolescence. LFC was below 10% for the pre-schoolers and increased to almost 20% at the end of adolescence. At the same time the estimated CAP-rate increased from 11 to 29% of ETST. The CAP-rate calculated from polysomnographic recordings was 25% for pre-schoolers and increased with progressing age (Parrino et al., 2012). At the age of approx. 15 years, the CAP-rate showed a peak (approx. 40%) and then slowly declined to approx. 35% at the age of 35. Neither the CAP-rate nor its local maximum were properly assessed by the estimated CAP-rate. The estimated CAP-rate generally underestimated the CAP-rate calculated from polysomnographic recordings. Furthermore, the more complex dynamics of the CAP-rate is not reflected by the estimated CAP-rate.

Very low frequency coupling as an indicator of REM/wake states increased with progressing age. REM and wakes states assessed from polysomnographic data show a constant [approx. 22% of sleep time (Ohayon et al., 2004)] or slightly decreasing amount of REM sleep [from approx. 25% to approx. 19% (Scholle et al., 2011)] during childhood and adolescence accompanied by a slight increase of wake after onset of sleep (from below 10 to 15%) (Ohayon et al., 2004). Hence, if these two numbers are added, VLFC seems to assess REM/wake states fairly well. The increase of VLFC with progressing age seems to be related to the increase of wake times after onset of sleep with progressing age.

Fragmented sleep or sleep disordered breathing is associated with the appearance of e-LFC (Thomas et al., 2007), which normally occurs around sleep onset, pre-REM sleep and at the junction of sleep cycles. Generally, the amount of respiratory disturbances during sleep increases from young adults to old age (Thomas et al., 2007; Pavlova et al., 2008). An increase of respiratory disturbances during childhood and adolescence has not been shown yet but seems to be plausible taking into account the present findings. The reliability of the approach of detecting sleep disordered breathing be mean of e-LFC is substantiated by the finding that a respiratory disturbance index based on LFC showed a good correlation with the respiratory disturbance index determined from polysomnographic recordings (Guo et al., 2011).

The amount of changes of sleep states between successive analysis windows, i.e., sleep fragmentation, increased with progressing age. A certain amount of changes of sleep state should be detectable because of the dynamics of sleep states. The present results suggest that the lower bound of fragmentation calculated by the present method should be approx. 20% on average.

As mentioned above, the social jetlag is responsible for the decline of the ETST. It should be investigated whether the decline of stable sleep and the increase of unstable sleep are associated with the delay of the body clock. I.e., sleep data acquired on free days should be investigated to control for effects caused by the social jet lag. Taken together, the data provide evidence that adolescent sleep is shorter, more unstable and, hence, poorer compared to sleep in early childhood. It remains to be clarified if this decline in sleep quality reflects normal maturational changes or if other factors such as, e.g., social jetlag contribute substantially to this finding. Furthermore, strategies should be developed that

make adolescents aware of the positive effects of sleep to enable better self-management with respect to sleeping habits.

AUTHOR CONTRIBUTIONS

DC, GS, and FE conceived the study. GS and ML planned and managed the study. ML recruited subjects and was responsible for the ECG recordings. DC performed the data analysis. DC and FE drafted the manuscript. All authors edited and approved the manuscript.

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