Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2016-0122.R2
Manuscript Type:	Article
Date Submitted by the Author:	19-Aug-2016
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Keyword:	Insulin Resistance, Cardiac Autonomic Tone, Women, Heart Rate Variability, Arterial Stiffness

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Manuscripts

Sex differences in associations between insulin resistance, heart rate variability and arterial stiffness in healthy women and men: a physiology study

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Word Count (text only): 3170, Tables: 2, Figures: 2, References: 45

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34 *Abstract*

35 Diabetes confers greater cardiovascular risk to women compared to men. Whether insulin
36 resistance-mediated risk extends to the healthy population is unknown. Measures of
37 insulin resistance (fasting insulin, homeostatic model assessment, hemoglobin A1c,
38 quantitative insulin sensitivity check index, glucose) were determined in 48 (56% female)
39 healthy subjects. Heart-rate variability was calculated by spectral power analysis and
40 arterial stiffness was determined using noninvasive applanation tonometry. Both were
41 measured at baseline and in response to angiotensin II infusion. In women, there was a
42 non-statistically significant trend towards increasing insulin resistance being associated
43 with an overall unfavourable HRV response and increased arterial stiffness to the
44 stressor, while men demonstrated the opposite response. Significant differences in the
45 associations between insulin resistance and cardiovascular physiological profile exist
46 between healthy women and men. Further studies investigating the sex differences in the
47 pathophysiology of insulin resistance in cardiovascular disease are warranted

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54 Key Words: insulin resistance, cardiac autonomic tone, human, women, heart rate
55 variability, arterial stiffness, cardiovascular, angiotensin II

Introduction

Insulin resistance is a stronger cardiovascular risk in women compared to men (Oterdoom et al. 2009). The risk factors for early cardiac autonomic impairment differ among diabetic women and men (Nolan et al. 2009) and fasting insulin level is a stronger determinant of arterial stiffness (Giltay et al. 1999) and cardiovascular outcomes (Oterdoom et al. 2009; Sarwar et al. 2007) in women than in men.

Impaired cardiac autonomic nervous system activity and increased arterial resistance, both known complications of insulin resistance, are each associated with greater cardiovascular risk (Vinik and Ziegler 2007; Yki-Jarvinen et al. 2007). The impact of insulin resistance on HRV, a measure of cardiac autonomic tone, differs by sex (Nolan et al. 2009) though whether these findings extend to the healthy population is unclear. Similarly, while individuals with impaired fasting glucose have higher stress measures of arterial stiffness, (Vasu et al. 2015) it is unknown whether this effect is modified by sex. We sought to determine the association between insulin resistance and HRV and arterial stiffness in healthy premenopausal women and men.

Methods

This study was approved by the University of Calgary Conjoint Health Research Ethics Board. Subjects provided informed written consent. All subjects were healthy normotensive, non-diabetic non-smokers and taking no prescription medications (including oral contraceptives). Subjects underwent a medical history, physical examination, and laboratory screening. Women were studied mid-menstrual cycle and

none were on the oral contraceptive. All subjects maintained a high-salt state (>150 mmol/day) for 3 days prior to the study to ensure maximum renin angiotensin system suppression (Shoback et al. 1983). Compliance with the high-salt diet was verified by measuring sodium excretion through a 24-h urine collection or a second morning void spot urine (Kawasaki et al. 1993). Each study commenced at 0800h following an overnight fast in a quiet, temperature-controlled room with subjects in a supine position. Blood pressure was measured via an automated sphygmomanometer (Dinamap, GE Healthcare, USA) every 15min. Subjects were infused intravenously with angiotensin II (AngII) ($3\text{ng/kg/min} \times 30\text{min}$; $6\text{ng/kg/min} \times 30\text{min}$).

Ambulatory electrocardiogram data were collected continuously (SEER MC recorder, GE Healthcare). HRV frequency measures were calculated according to standard methods (MARS version 7, GE Healthcare). Frequency domain parameters were derived using power spectral analysis, where a combination of sympathetic and vagal autonomic nervous system (ANS) power was represented by the low-frequency (LF; ms^2) band within the range of 0.04–0.15 Hz, and vagal power was represented by the high-frequency (HF; ms^2) band within the range of 0.15–0.40 Hz, both of which were expressed in normalized units (nu) to account for changes in total power. The LF:HF ratio component was calculated by comparing crude LF and HF parameters, representing total cardiosympathovagal balance. Impaired cardiac autonomic tone, a known complication of insulin resistance, is associated with increased mortality (Vink et al. 2007). HRV, represents a balance between cardiac sympathetic (LF) and parasympathetic tone (HF) (vagal tone), with greater variability in heart rate considered a marker of a healthy autonomic nervous system (Karayannis et al. 2012).

The bPWV and AIx were measured noninvasively with applanation tonometry (Millar Instruments, Houston, TX) and commercially available acquisition and analysis software (Version 8 SphygmoCor; AtCor Medical, Sydney, Australia) every 15 min, as previously described (Laurent et al. 2006). Subjects were studied in the supine position using a standard cuff placed on the right arm. Two readings were taken and recorded at each time point by the same registered nurse and the mean was reported. The peripheral vascular stiffness was represented by bPWV, while the systemic vascular stiffness was represented by AIx. Aortic augmentation is a validated measure of central vascular stiffness and is also associated with adverse cardiovascular outcomes (Blacher et al. 1998; Kelly et al. 2001; Livingstone et al. 2013; Oparil et al. 2006; Smulyan et al. 2003). Serum insulin levels were determined by chemiluminescent immunoassay (Abbott Diagnostics, USA), HbA1c levels by turbidometric immunoassay and serum glucose by hexokinase UV colorimetric (both Roche Diagnostics; Germany). Homeostatic Model Assessment Insulin Resistance (HOMA-IR) was calculated as $[(\text{Fasting Glucose}(\text{mmol/l})) \times (\text{Fasting Insulin}(\mu\text{mol/l}))] / 22.5$ (Wallace et al. 2004) and Quantitative Insulin Sensitivity Check Index (QUICKI) as $1 / [\log(\text{Fasting Insulin}(\mu\text{mol/l})) + \log(\text{Fasting Glucose}(\text{mg/dl}))]$ (Katz et al. 2000).

The exploratory primary analysis examined the association between parameters of insulin resistance (fasting insulin, HOMA-IR, HbA1c, QUICKI, glucose) and HRV and arterial stiffness at baseline and in response to 60min AngII challenge, stratified by sex. Baseline and response to AngII measures were compared using Student t-test. Frequencies were compared by χ^2 tests. A stepwise selection multivariate linear regression was applied to determine the relative contributions of covariates (for HRV:

sex, age, body mass index (BMI), MAP, heart rate, high-density lipoprotein (HDL), low-density lipoprotein (LDL), serum 25-hydroxyvitamin D, serum estradiol (women), testosterone (men), and where appropriate, baseline LF, HF, or LF:HF; for arterial stiffness: sex, age, BMI, MAP, HDL, LDL, serum 25-hydroxyvitamin D, uric acid, and where appropriate, baseline AIX and bPWV). All model assumptions were tested and met. Analyses were performed using Stata (version 10.0, STATA Corp. College Station Tx) with $\alpha=0.05$.

Results

The characteristics of the 48 healthy subjects (56% female, 75% Caucasian) enrolled have been published previously (Mann et al. 2012; Ramesh et al. 2015; Samimi et al. 2014) and are outlined in Table 1. All subjects had normal measures of insulin resistance (Chen et al. 2005), baseline HRV (Nunan et al. 2010) and baseline arterial stiffness (Laurent et al. 2016).

Neither women nor men demonstrated any association between any measure of insulin resistance and measures of baseline HRV or measures of baseline arterial stiffness. However, on univariate analysis, there was a non-significant trend towards an increased LF response to AngII ($r=0.27$, $p=0.1$; $p=0.07$ on multivariate analysis) and a more blunted HF response ($r=-0.24$, $p=0.16$; $p=0.07$ on multivariate analysis) with increasing fasting insulin levels in women. This was reflected in a non-significant increase in overall LF:HF ($p=0.1$ vs baseline). When adjusted for covariates, this increased overall cardiosympathovagal balance approached significance ($p=0.05$ vs baseline) (Figure 1). Men demonstrated the reverse association between insulin levels and

the LF ($p=0.02$) (women vs men LF, $p=0.06$) and HF ($p=0.009$) (women vs men HF, $p=0.07$) responses to AngII (women vs men LF:HF response: $p=0.049$) when adjusted for all other covariates (Figure 1).

Similarly, men showed a negative relationship between HOMA-IR and LF ($p=0.05$) response to Ang II challenge and a significantly positive association with HF ($p=0.02$). While not statistically significant, an opposite relationship was observed in women with a positive trend between HOMA-IR and the LF response to AngII ($p=0.1$) on multivariate analysis and a negative trend with the HF ($p=0.15$). No associations were observed between HbA1c and measures of HRV in response to AngII challenge in either sex.

In women, increasing insulin sensitivity as demonstrated by QUICKI measurements was associated with a trend towards decreasing LF ($p=0.1$) and increasing HF ($p=0.1$) responses to AngII, while men showed the opposite association (LF, $p=0.016$; $p=0.003$ vs female response; HF, $p=0.013$; $p=0.002$ vs. female response) on multivariate analysis.

Women did not demonstrate any association between fasting glucose and any HRV measure in response to AngII challenge. Men showed no association between glucose and the change in LF in response to AngII but glucose was associated with the HF response to AngII ($r=0.34$, $p=0.046$) and this was statistically different from women ($p=0.018$). Results of the heart rate variability data are summarized in Table 2.

Arterial Stiffness response to AngII

As anticipated, all subjects demonstrated a significant increase in measures of arterial stiffness (Table 2), with no differences between the female and male responses to AngII (AIX: $p=0.41$; PWV, $p=0.29$, women vs men).

In response to AngII, however, women demonstrated a positive association between fasting insulin levels and the AIX response ($r=0.37$, $p=0.023$), a response not observed in men (women vs men response, $p=0.07$). Similarly, HOMA-IR was positively associated with the AIX response to AngII after adjustment for covariates in women ($p=0.03$), but not men (women vs men response, $p=0.07$) (Figure 2). There were no associations between any of the other measures of insulin resistance and the AIX response to AngII in either sex. No association was observed between measures of insulin resistance and bPWV in either sex. There was no statistical significant difference between women and men in MAP after AngII infusion. In addition no association was observed between measures of insulin resistance and delta MAP in women or men.

Discussion

Our findings were as follows: 1) in women, increasing insulin resistance was not associated with a change in HRV in response to a physiologic stressor, whereas men showed a decrease in LF, a marker of cardiosympathetic activity and an increase in HF, a marker of protective cardiovagal input with increasing levels of insulin resistance; 2) increasing insulin resistance was associated with a greater increase in stress-related arterial stiffness in women but not men. Our results add to the mounting evidence that important sex differences exist in the pathophysiology by which insulin resistance affects cardiovascular risk.

Why sex modifies the cardiovascular risk profile associated with insulin resistance is not clear (Shaw et al. 2006). A meta-analysis of 37 prospective cohort studies of type 2 diabetes and fatal coronary heart disease with a total of 447,064 patients demonstrated that the relative risk for fatal coronary heart disease associated

with diabetes was 50% higher in women compared to men (Huxley et al. 2006). Compared to healthy subjects, diabetes was associated with a four- to fivefold higher coronary artery disease rate among women, but not men (Kalyani et al. 2014). However, investigation of the impact of diabetes or insulin resistance on the pathophysiology leading to CVD is extremely limited. Impaired glucose tolerance is more common among women, whereas men are more likely to demonstrate impaired fasting glucose (Cowie et al. 2006), though how this may play into poorer cardiovascular outcomes in women is not clear. Impaired glucose tolerance enhances non-enzymatic glycation and cross-linking of collagen (Brownlee et al. 1988), which may thus predispose women to greater arterial stiffness, though this remains speculative.

Studies examining sex differences in diabetic patients and HRV suggest that significant differences exist between the sexes in the onset and severity in the development of autonomic nervous system dysfunction (Nolan et al. 2009; Sengstock et al. 2005). Nolan et al. 2009 reported that impaired HRV in women was associated with the age at which the diagnosis of diabetes was made, whereas in men the development of HRV abnormalities was associated with the duration of diabetes (Nolan et al. 2009). Previous studies have suggested that differences in compliance and treatment intensity of glycemic control may explain the sexual discrepancy in favour of men (Kramer et al. 2012), but this is unlikely given that none of the healthy subjects in the present study were on treatment.

Endogenous sex hormones appear to play a role in mediating risk in the setting of insulin resistance, with elevated levels of testosterone having opposing effects in men and women (Ding et al. 2006). In older women, higher free testosterone levels were

associated with greater insulin resistance (Oh et al. 2002). A meta-analysis examining the role of sex hormones in the risk of developing diabetes reported a higher risk of type 2 diabetes among women with increased testosterone levels, with the opposite association in men (Ding et al. 2006). It was postulated that the elevated testosterone at the levels increases adipose tissue in women and therefore may subsequently increase in insulin resistance, however the exact mechanism remains unclear (Lovejoy et al. 1996). Similarly, low levels of sex-hormone binding globulin were associated with development of diabetes in women but not in men, though a nested case-control study reported that low circulating levels of sex hormone-binding globulin were a strong predictor of the risk of type 2 diabetes in both women and men (Ding et al. 2006).

The role of circulating estrogen levels as a risk for developing diabetes in women and men is less clear. In a meta-analysis, estradiol levels were elevated among men and postmenopausal women with diabetes compared with controls, however there was no sex-dimorphism between the groups (Ding et al 2006). There was insufficient prospective data or studies to determine any additional association between estrogen levels and insulin resistance (Ding et al. 2006). In a study of post-menopausal women, physiological doses of estradiol did not alter arterial stiffness or insulin sensitivity (Vehkavaara et al. 2000). However, interpretation of the effects of estrogens on insulin resistance and markers of cardiovascular disease remains challenging due to differences in age of study populations, timing of menopause, and the type and route of administration of postmenopausal hormone therapy (Grodstein et al. 2003). Of note, endogenous sex hormone levels were accounted for in our analysis. Furthermore, none of the women in

the study were ingesting the oral contraceptive and all women were studied at the same phase of the menstrual cycle.

Fasting insulin is a strong predictor of cardiovascular disease in women. A meta-analysis suggested that elevated fasting insulin concentrations conferred a greater cardiovascular risk in women (Sarwar et al. 2007). In a study of 6916 non-diabetic participants, every doubling of fasting insulin in women increased the risk of a cardiovascular event by 50% even after exclusion of those with impaired fasting glucose, an association not observed in men (Oterdoom et al. 2009).

Both depressed HRV and increased arterial stiffness are validated markers of cardiovascular risk. Increased HOMA-IR is an independent risk factor for HRV abnormalities in a young, non-diabetic population (Pal et al. 2013). In a systematic review, HRV was reduced in women with metabolic syndrome with insulin resistance highlighted as the potential principal factor responsible, though findings in men were inconsistent (Stuckey et al. 2014). The vasodilating effect of insulin is blunted in subjects with insulin resistance (Tamminen et al. 2002), suggesting another possible mechanism by which increased insulin resistance augments cardiovascular risk. With the exception of one male subject with elevated fasting insulin levels, none of the subjects in our study had evidence of the metabolic syndrome, which may explain the lack of association observed between insulin resistance and baseline measures of HRV and arterial stiffness. We speculate that in the earliest stages of insulin resistance only a stressor may uncover abnormalities in cardiac autonomic and arterial tone as observed in our study.

Due to the cross-sectional nature of our study, we cannot demonstrate directionality of the association nor comment on causality; it is possible that

dysfunctional HRV or arterial stiffness caused an increase in insulin resistance in women, although this would not explain why the same phenomenon did not occur in men. While the study of healthy non-diabetic subjects limits the generalizability of the results, by studying a normotensive, healthy population, we aimed to examine the impact of insulin resistance and sex on HRV and measures of arterial stiffness while minimizing confounding factors. Second, we attempted to minimize the effect of sample size and intra-individual variability by utilizing a homogenous study group and careful pre-study design. We ensured that all participants were ingesting similar amounts of salt to ensure maximum RAS suppression, that no female participant was ingesting oral contraceptives, and that all female subjects were studied during the same stage of the menstrual cycle, during the follicular (low estrogen) stage to control for the effect of estrogen on blood pressure and the RAS. In addition, all subjects were studied at the same time of the day, while resting in the supine position in a warm, quiet room after an 8-h fast. We measured insulin resistance using the HOMA model rather than a hyperinsulinemic-euglycemic clamp. However, the HOMA model is a validated clinical and epidemiological tool (Wallace et al. 2004). Furthermore, measures of insulin resistance were collected at baseline and we were thus unable to account for any potential changes in insulin sensitivity as a result of an Ang II infusion. However a study examining the effect of a concurrent hyperinsulinemic clamp and Ang II infusion in healthy non-diabetic subjects did not report any change to overall insulin sensitivity (Jonk et al. 2010). In a study of healthy men and women, it was reported that random error represented a limited part of the between-subject variability; therefore observed differences between individuals mostly reflect differences in the subjects' error-free value rather than random error (Pinna

et al. 2007). Furthermore, exclusion of one male subject who had a significantly higher baseline serum insulin level showed a similar non-significant trend association between insulin resistance and changes in LF and HF. This subject's BMI of 32 may contribute to a higher circulating insulin level, though other measures of insulin resistance including hemoglobin A1c (5.4%), fasting glucose (5.6) HOMA-IR (4.98) and QUICKI (0.30) were within the normal non-diabetic range and consistent with other studies of non-diabetic obese males (Chen et al. 2005). AIx assesses systemic arterial stiffness, whereas PWV is a measure of the arterial stiffness between two recording sites (Kim and Braam, 2013). PWV determined from central arteries such as the aorta, carotid or femoral arteries is considered the "gold standard" instead of the carotid-radial method utilized in this study. However, the use of more peripheral arteries provided a less invasive method to measure arterial stiffness (Laurent et al. 2016, Kim et al. 2013). Measurement of arterial stiffness through brachial-ankle PWV has been demonstrated to provide similar predictive information as that determined from central arterial stiffness in young healthy adults, (Sugawara et al. 2005, Tsuchikura et al. 2010) similar to the population in our study. In a study of healthy males which compared central aortic pulse wave transient time measured by MRI to arterial stiffness measured by brachial PWV, there was close correlation between the two measurement techniques. (Rezai et al. 2013). Finally, measurement of the augmentation index (AIx) measured centrally and peripherally at the brachial site has been compared and demonstrated to have a close correlation (Rezai et al. 2011).

Conclusion

The results of this study suggest that insulin resistance confers a sex-dependent unfavourable cardiac ANS response to a physiologic stressor. Further studies investigating the pathophysiology of insulin resistance in CVD in women are warranted.

Acknowledgements

Author Contributions: LAR researched data, wrote manuscript; JMM researched data, contributed to discussion, reviewed/edited manuscript; MCM researched data, reviewed/edited manuscript; SR researched data, reviewed/edited manuscript; BRH contributed to discussion, reviewed/edited manuscript; DR contributed to discussion, reviewed/edited manuscript; DYS researched data, reviewed/edited manuscript; SBA researched data, contributed to discussion, reviewed/edited manuscript). LAR and SBA take responsibility for the contents of the article. This study was presented in abstract form at the Canadian Society of Nephrology Annual Meeting in Montreal, Canada in April 2015.

This study was funded by an Establishment Grant from Alberta Innovates – Health Solutions. MCM is supported by the Canadian Institutes of Health Research. SR is supported by the Roy and Vi Baay Chair Doctoral Scholarship. DR, BRH and SBA are supported by Alberta Innovates – Health Solutions. JMM, BRH, DR and SBA are supported by a joint initiative between Alberta Health and Wellness and the Universities of Alberta and Calgary. Funding sources had no role in the design, conduct, or reporting of this study.

The authors declare they have no competing interests.

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FIGURE 1. Heart rate variability response to AngII as a function of fasting insulin level, by sex. __

PANEL A – Low Frequency verses Insulin Level * $p = 0.07$ vs. Response to Women

PANEL B – High Frequency verse Insulin Level * $p = 0.06$ vs. Response to Women

PANEL C – Low Frequency:High Frequency Ration verses Insulin Level * $p < 0.05$ vs. Response to Women

FIGURE 2. Arterial stiffness response to AngII challenge as a function of HOMA-IR, by sex.

* $p = 0.07$ vs Female Response

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Table 1. Baseline Characteristic: Baseline clinical and laboratory characteristics of women versus female subjects.

Characteristic	Women (<i>n</i> = 27)	Men (<i>n</i> = 21)
Age (years)	33 ± 2	40 ± 3
Caucasian (%)	71%	81%
BMI (Kg/m ²)	24 ± 0.7	26 ± 1
Mean Arterial Pressure (mmHg)	75 ± 4	88 ± 3*
Systolic Blood Pressure (mmHg)	105 ± 4	121 ± 4*
Diastolic Blood Pressure (mmHg)	62 ± 3	72 ± 3*
Testosterone (nmol/l)	1.5 ± 0.1	17.3 ± 5*
Estradiol (pmol/l)	402 ± 65	100 ± 35*
Progesterone (nmol/l)	8.9 ± 3	1.7 ± 0.7*
Triglycerides (mmol/l)	0.66 ± 0.05	0.96 ± 0.1*
LDL (mmol/l)	2.1 ± 0.1	2.47 ± 0.13
HDL (mmol/l)	1.55 ± 0.06	1.23 ± 0.06*
Fasting Glucose (mmol/l)	4.5 ± 0.1	4.7 ± 0.1
Insulin (pmol/l)	39 ± 3	43 ± 6
HbA1c % (mmol/mol)	5.5 ± 0.05 (37)	5.5 ± 0.1 (37)
HOMA-IR	1.15 ± 0.1	1.33 ± 0.2
QUICKI	0.38 ± 0.05	0.38 ± 0.01

All data are expressed as mean ± SE unless otherwise indicated.
Abbreviations: BMI, body mass index; LDL, Low-Density Lipoprotein; HDL, High-Density Lipoprotein; HbA1C, Hemoglobin A1c; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; QUICKI, Quantitative Insulin Sensitivity Check Index
* P<0.05 vs women

Table 2. Heart rate variability, arterial stiffness and blood pressure at baseline and in response to Angiotensin II infusion challenge in women and men.

	Women (n = 27)		Male (n = 21)	
Heart-Rate Variability	Baseline	Response to Ang II Challenge	Baseline	Response to Ang II Challenge
LF (ln ms ²)	6.84 ± 0.21	6.89 ± 0.19	7.01 ± 0.16	7.15 ± 0.15
HF (ln ms ²)	6.33 ± 0.21	6.29 ± 0.24	5.86 ± 0.24	5.88 ± 0.22
LF (nu)	59.57 ± 2.58	61.69 ± 2.05	72.38 ± 2.76	75.35 ± 2.08*
HF (nu)	37.29 ± 2.69	35.34 ± 2.09	25.61 ± 2.57	22.96 ± 1.97*
LF:HF (nu)	1.35 ± 0.08	1.39 ± 0.08	1.87 ± 0.15	1.96 ± 0.13*
Arterial Stiffness				
AIx	8.63 ± 2.57	17.35 ± 2.47	2.55 ± 3.67	14.29 ± 2.72
PWV	7.22 ± 0.2	8.74 ± 0.4	7.87 ± 0.2	9.39 ± 0.3
Blood Pressure				
Systolic (mmHg)	106 ± 4	130 ± 3	119 ± 3	140 ± 6
Diastolic (mmHg)	62 ± 3	80 ± 2	71 ± 2	98 ± 2

All data are expressed as mean ± SE unless otherwise indicated. Δ values in parentheses indicate mean change from baseline. * p<0.05 versus responses to women.

Abbreviations: Ang, angiotensin; LF, low-frequency; HF, high-frequency; LF:HF, high-frequency-low-frequency ratio; AIx, aortic augmentation index