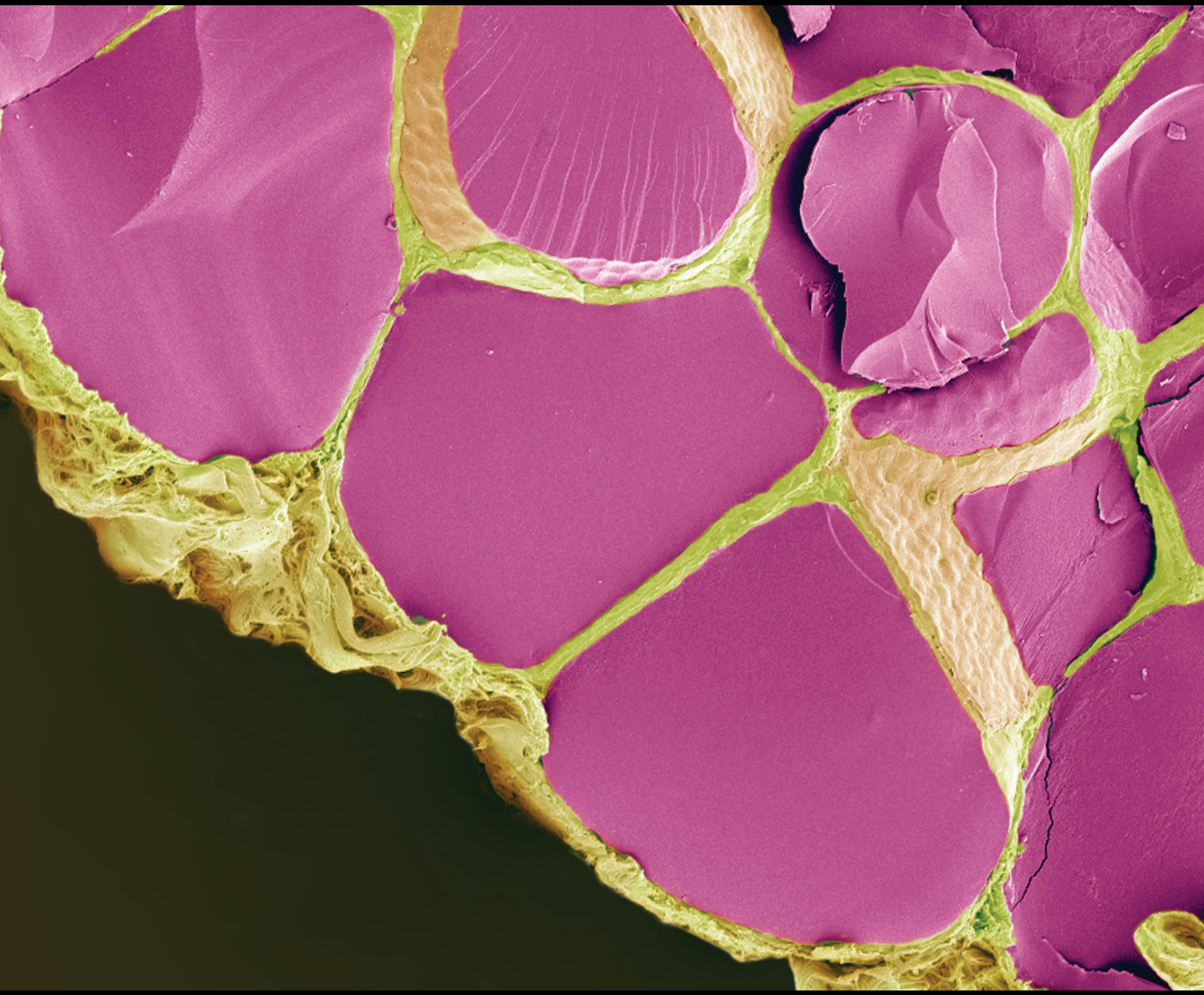
International Journal of Endocrinology

Regulation of Cardiovascular Metabolism by Hormones and Growth Factors

Guest Editors: Cristian Ibarra, Sergio Lavandero, and Manuel Estrada



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Editorial Board

Contents

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Editorial

Regulation of Cardiovascular Metabolism by Hormones and Growth Factors

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Currently, metabolic disorders are a cardinal focus of research given their impact in public health problems worldwide. The main risk factors for death in people suffering metabolic disorders are cardiovascular diseases. Metabolic changes are important settings for cardiac cells under these conditions because the cardiovascular system must maintain energy balance to preserve work output and efficiency of the heart. Cardiovascular system is regulated by hormones which control, adjust, and remodel heart metabolism.

The role of hormones and growth factors in modulating cardiovascular function impacts different areas of research, especially considering that atypical plasma levels together with variable effects at cellular level are hallmarks of several medical conditions such as metabolic syndrome, obesity, diabetes, ischemic heart disease, and heart failure. Hormones and growth factors produce integrative systemic effects that involve ligand/receptor interactions, generations of second messengers, activation of intracellular signaling cascades, posttranslational modification of proteins, and gene expression. The implications of hormone and growth factor effects encompass the diagnosis, clinical evaluation, and pharmacological intervention. Several studies on metabolic effects of hormones and growth factors in the cardiovascular system are being translated into therapeutic applications.

In this issue E. Ostu et al. show the heart as a neuroendocrine gland that interacts with both hormones and cytokines. The authors discuss the current evidence about the dynamic communication between the heart and other organs involved in cardiovascular homeostasis. The exploration of microvascular function is important as predictive tool and as prognosis of cardiovascular risk and progression of heart failure. This paper describes the present knowledge on whether hormones and cytokines influence microvascular function and coronary flow reserve. The work is an interesting contribution to understand the pathological mechanisms of microvascular dysfunction which could further benefit patients suffering metabolic and cardiovascular diseases.

The incidence of cardiovascular mortality, including sudden death, is higher in men than in women. Obesity, metabolic syndrome, and type 2 diabetes mellitus are major risk factors for cardiovascular disease. Here, L. Skrgatic et al. review publications from the late 1980s to the present to infer whether polycystic ovary syndrome is a cardiometabolic risk factor according to its relationship to several intrinsic factors that are known to produce these metabolic disturbances. Meta-analysis and clinical evidence correlate low testosterone plasma concentrations with metabolic disorders and cardiovascular damage, but safety concerns have been raised over testosterone replacement therapy as an association between myocardial infarction and testosterone replacement therapy in men has been suggested. W. Reilly et al. carried out a large, retrospective study in a multicenter practice center

for testosterone treatment. This analysis in patients treated with testosterone did not correlate with high incidence of myocardial infarction. Further studies should be conducted in this field in order to elaborate safe therapeutic replacement protocols.

Adipose tissue is an endocrine gland and key controller of body metabolism that interlinks with cardiovascular system. Early, simple, and fast biomarkers determining the association between altered focal point of adipose tissue and cardiovascular risk factors are useful for handling large scale and nutritional interventions and early therapeutic. The work of G. Curic et al. shows the relationship between subcutaneous and visceral adipose tissues with incidence of coronary artery disease in cohort of patients assessed at the cardiology unit. Using low cost anthropometric measures obtained from coronary artery disease and noncoronary artery disease patients, the authors found that anthropometric measures and subcutaneous adipose tissue have nonlinear relationship with the expansion of epicardial adipose tissue which is linked to coronary artery disease. Thus epicardial adipose tissue thickness and anthropometric measures have similar coronary artery disease predictive value.

Several studies have shown that consumption of energy drinks has short term impact on cardiac contractility. The cardiovascular and metabolic effects of these beverages have been associated with a high intake of caffeine, including high heart rate, palpitations, and increases in blood pressure, whereas hyperglycemic effects are associated with high sugar content. However, high concentrations of caffeine do not fully explain these effects; in this sense the paper of A. Panduric et al. explores the activation of the adrenergic system as a potential mechanism for cardiovascular and hyperglycemic effects produced by elevated energy drink intake. Through determination of heart rate, arterial blood pressure, blood glucose, adrenaline, and noradrenalin plasma levels before and after energy drink intake, the authors argue about positive cognitive functions and effect on cardiovascular and respiratory system at rest and during exercise by increasing activity of the sympathetic nervous system being due, in part, by adrenergic activation.

A large number of studies have shown that altered metabolic action of hormones and growth factors correlates significantly with the incidence of cardiovascular disease. These studies suggest the molecular basis for an integrated model of energy unbalance produced in cardiovascular system and hormone action and function. Furthermore, hormone interventional studies have shown an improvement in these cardiovascular risk factors.

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Research Article

Adrenergic System Activation Mediates Changes in Cardiovascular and Psychomotoric Reactions in Young Individuals after Red Bull© Energy Drink Consumption

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Objectives. To assess the effect of Red Bull© on (1) blood glucose and catecholamine levels, (2) cardiovascular and respiratory function changes before, during, and after exercise, (3) reaction time, (4) cognitive functions, and (5) response to mental stress test and emotions in young healthy individuals ($N = 38$). *Methods.* Heart rate (HR) and arterial blood pressure (ABP), blood glucose, adrenaline, and noradrenalin plasma levels were measured before and after Red Bull© intake. Participants were subjected to 4 different study protocols by randomized order, before and 30 minutes after consumption of 500 mL of Red Bull©. *Results.* Mean ABP and HR were significantly increased at rest after Red Bull© intake. Blood glucose level and plasma catecholamine levels significantly increased after Red Bull© consumption. Heart rate, respiration rate, and respiratory flow rate were significantly increased during exercise after Red Bull© consumption compared to control condition. Intake of Red Bull© significantly improved reaction time, performance in immediate memory test, verbal fluency, and subject's attention as well as performance in mental stress test. *Conclusion.* This study demonstrated that Red Bull© has beneficial effect on some cognitive functions and effect on cardiovascular and respiratory system at rest and during exercise by increasing activity of the sympathetic nervous system.

1. Introduction

Although the consumption of energy drinks is somewhat declining [1, 2] compared to previous decades [3], still a lot of young people, athletes, and especially college students consume energy drinks before practicing, while studying, and before exams because they believe that this improves their performance and memory [4]. Red Bull© is among the most popular energy drinks with annual global sales of several billion dollars [5]. Red Bull© is sold under the slogan "gives you wings" which suggests that its consumption will provide the consumer with more energy and enhanced performance, both mentally and physically. The manufacturers credit these physiological and neurological benefits to Red Bull©'s active ingredients that include: caffeine (approximately 32 mg/dL), glucuronolactone (approximately 240 mg/dL), and taurine (approximately 400 mg/dL), as well as B vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine hydrochloride, biotin, inositol, and cyanocobalamin) and sugars (glucose and saccharose) [6].

In terms of physiological effects of Red Bull©, most of the documented changes have focused on the subjects' blood pressure (BP) and heart rate (HR) at rest [7, 8]. The results of these studies are inconclusive for both initial assumed

effects of the consumption of energy drink, raising the BP and lowering the HR [4, 7–9]. These contradictory results may occur due to opposing effects of two active ingredients of Red Bull©: caffeine and taurine. Caffeine is characterized as a central nervous system stimulant promoting activation of the sympathetic adrenal-medullar system that leads to immediately increased blood pressure and peripheral vascular resistance [6, 10, 11]. On the other hand, taurine, a sulfur-containing amino acid, seems to suppress sympathetic nervous system stimulation by modulating cyclic nucleotide content in heart cells [12, 13]. Limited number of controlled studies on Red Bull© effects on exercise performance has given inconsistent results, as well. Most of the studies on young athletes indicate that energy drinks improve endurance performance [14], although there is less support for its ergogenic properties during strength- and power-based exercise dependent upon oxygen-independent metabolism [15, 16]. Observed ergogenic benefits of energy drinks are likely attributable to caffeine and glucose content [6, 17]. The notable findings of some recent studies are that nowadays caffeine content in energy drinks is comparable to coffee beverages in Europe and USA [1, 18].

Even though Red Bull© is promoted as energy drink that improves mental alertness and performance, the number of studies that researched this topic is limited. Alford et al. found that Red Bull© improved reaction times, alertness, and concentration and that improved performance on an immediate recall memory test in young healthy students [7]. The other study has shown that Red Bull© has beneficial effect on cognitive performance and mood in graduate student population [19]. On contrary, Bichler et al. demonstrated that Red Bull© energy drink has no effect on short term memory in young college students [4]. The results of one recent study indicated that energy drink consumption decreased reaction times on behavioral control tasks, increased subjective ratings of stimulation and decreased ratings of mental fatigue [20]. The number of young people taking energy drinks is still high, and the results of studies that have investigated the effects of Red Bull© intake and the possible role of adrenergic system activation and other mechanisms through which its active ingredients work are inconsistent.

Since activation of sympathoadrenergic system is crucial in cardiovascular and psychomotoric reactions and Red Bull© has ingredients that could modulate levels of adrenergic hormones, the aim of our study was to determine the involvement of adrenergic system in effects contributed to Red Bull© consumption on (1) HR, BP, blood glucose, and plasma catecholamines levels at rest, (2) cardiac, and respiratory changes before, during, and after moderate exercise, (3) reaction time to audio stimuli, (4) cognitive functions, and also (5) response to mental stress (distracting three digit numbers) test and emotions in young healthy individuals.

2. Methods

2.1. Study Population. Thirty-eight young healthy medical students were recruited for the study by advertisement at the Faculty of Medicine Josip Juraj Strossmayer, University of Osijek. All volunteers were self-described as healthy, with no history of cardiovascular, urinary, digestive, or metabolic diseases. All subjects were familiar with Red Bull© and most of them had previously consumed this energy drink. Written informed consent was obtained from each subject. The study protocol and procedures conformed to the standards set by the latest revision of the *Declaration of Helsinki* and were approved by the Ethical Committee of Faculty of Medicine, University of Osijek.

2.2. Study Protocol. All subjects were instructed not to consume alcohol or energy drinks 7 days prior testing. They all were fasting 12 h prior to the onset of the experiment. Subject height and weight were measured to determine body mass index (BMI), as well as the extent of hips and waist to determine waist-to-hip ratio (WHR). BP and HR were measured at the beginning of the experiment after 15 minutes rest in seated position. Semiautomatic oscillometric monitor (OMRON) was used. Final values of BP and HR were mean of three repeated measurements. Intravenous cannula was inserted into a vein and a venous blood sample was taken after 30-min resting in supine position. A separate group of 9 participants stay resting in supine position during whole study visit and blood samples for plasma catecholamine levels were taken after 30 minutes resting in supine position (before Red Bull© consumption) and 15, 30, 45, and 60 minutes after 500 mL of Red Bull© consumption. This was necessary due to great variability in catecholamine levels when person in moving and results of the measurements would be inconclusive for the observed effects. Prior to Red Bull© consumption, other 29 participant were assigned to 4 different study protocols (described below) by randomized order. After control protocol subjects were instructed to drink 500 mL of Red Bull© and after 30 minutes of resting in supine position, all tests, including BP, HR, blood tests, and each of 4 study protocols were repeated by randomized order.

2.2.1. Protocol 1: Influence of Red Bull© Consumption on Cardiac and Respiratory Changes during Moderate Exercise. The Harvard Step Test (HST) was used to detect cardiac and respiratory changes during moderate exercise. The HST is performed in a manner that the subject steps up and down into the default beat for three minutes on the 45 centimeters high step bench. Male participants had stepped on the bench 24 times in one minute (24/per min), while female participants had stepped on the bench 22 times in one minute (22/per min). For determining the rate, metronome was used and was set at 96 beats per minute for male subjects and 88 beats per minute for female subjects. Each tick marked a movement with subject's leg. At the START signal participant stepped up, first with one foot on the bench, and then another, and after stretching legs and straightening back immediately stepped down, gliding with leg which first stepped up. Stepping up and down always started with the same leg (with maximum two changes during the test). In one hand subject held air flow transducer and temperature sensor was attached to his other hand. The physiological functions of the subject were measured during seven minutes, first minute at rest, next three minutes during stepping up and down, and last three minutes at rest after exercise.

Three parameters were measured during this study protocol: HR, air flow, and skin temperature (ST). These parameters were assessed with three electrodes attached to subject's chest, air flow transducer into which subject breathed with clamp on his nose and skin temperature sensor mounted on his finger which were all connected to BIOPAC device (BIOPAC Systems Inc., Goleta, CA, USA). For data processing BIOPAC software was used (BIOPAC Systems Inc., Goleta, CA, USA). All measurements were performed by a single trained operator.

2.2.2. Protocol 2: Influence of Red Bull© Consumption on Reaction Time. Reaction time (RT) was measured by BIOPAC device and software (BIOPAC Systems Inc., Goleta, CA, USA). Measurement was performed in quiet room. Subject was sitting eyes closed with headphones and with push-button device in his hand which he pressed on sound signal. The first part of the test consisted of series of ten beeps that occurred in randomized time intervals, and second part in which beeps occurred in fixed time intervals. Both tests lasted for one minute, with one minute pause between tests.

2.2.3. Protocol 3: Influence of Red Bull© Consumption on Cognitive Functions. All cognitive tasks were assessed by randomized order in quiet room by a single trained operator. For cognitive function assessment following tests were used.

Auditory Verbal Learning Test (AVLT). To examine global cognitive functions, the alternate form of Rey's Auditory Verbal Learning Test (AVLT) published by Lezak in 1995 was utilized [21]. This cognitive task has been shown to reflect not only specific verbal learning and memory, but also global cognitive functions [22–24]. Two lists (A and B) of 15 words were used, for repeated tests parallel lists of words were available. Five presentations of the list A were given, each followed by attempted recall. The first trial of the AVLT is a measure of immediate memory. After five trials a second 15-word list (list B, distraction list) was read and followed by a recall trial of this list and then another recall trial of the list A (measure of delayed recall). After a delay interval of 30 minutes and no further presentations of the lists, delayed verbal memory was assessed by recall trial of the list A.

Fluency Tasks. Each participant underwent three types of verbal fluency tests: phonemic, semantic, and ideational [21]. Each test consisted of trials (90 second duration) directing participants to generate as many words as possible (a) that began with a particular letter ("F," "A," or "S") excluding proper names and variations of the same word, (b) that were exemplars of an unprimed semantic category (e.g., animals), or (c) that were exemplars of an unprimed ideational category (e.g., metal objects). The final score was given as number of correct words generated during first 60 seconds.

Auditory Digit Span Task (ADST). The digit span task is one of Wechsler's group of intelligence tests used to access the immediate and working memory function [25, 26]. A list of random numbers was used and read out loud at the rate of one per second. The participants were asked to repeat an increasing array of numbers in backwards (working memory) or forward order (immediate memory) until they committed an error.

d2 Attention Loading Test. This test was constructed by German psychologist Rolf Brickenkamp in 1962. We have used an updated and revised form published in 1994. The d2 attention loading test is one of the general competence tests that have been used for measuring attention and ability to concentrate, sustained attention. In the present study, an official form of the attention loading test was used (Copyright by Hogrefe, Verlag GmbH & Co., KG Göttingen, 1994). It is a timed test composed of the letters "d" and "p" with one, two, three, or four dashes arranged either individually and/or in pairs above and below the letter. There are 14 lines of 47 characters each. Subjects are given 20 seconds to scan each line and cross all the "d" marked with two dashes. The test analysis appoints quality and quantity of processed data, in general and during the time of the test. Several variables were used for the test analysis, as follows: TN: total number of processed data, E: total errors (OE: omitted errors + RE: replacing errors), TN-E: quantitative measure defined by total number of processed data minus number of total errors, MC: mean concentration, qualitative measure calculated as number of correctly processed data reduced for number of omitted errors. MC is a variable that protects from possible cheating on the test (random data processing cannot increase MC).

2.2.4. Protocol 4: Influence of Red Bull© Consumption on Emotional Status and Mental Stress Response

Mental Stress Test. Mental stress test (MST) was used to provoke subject's emotional response. Stressor was 2-minute long arithmetic test in form of, at first sight, simple arithmetic operation (e.g., 345 – 193 = 152). Subject's task was to answer is the operation correct or incorrect with time limit of 3 seconds for each task. If subject did not offer answer, it was recorded as incorrect. After each answer, subject got feedback information if the answer was correct or not by different sound signals. In 2 minutes subject got 40 different arithmetic tasks, and after the test got information of his success. With 100% correct answers subject got 400 points. To pass the test it was necessary to respond correctly on 80% of the tasks and get 320 points. Subject was informed about his success on the test. After MST participants were subjected to Aggression Questionnaire and Beck Anxiety Scale.

Aggression Questionnaire. The Aggression Questionnaire AG-87 is intended to measure the tendency toward aggressive behavior in provoking situations, to measure impulsive aggression. Questionnaire A-87 consists of 15 items of different situations with five possible responses. The possible responses or reactions are the five most frequent forms of aggressive responses: (a) *verbal manifest aggression* (VM); (b) *physical manifest aggression* (PHM); (c) *indirect aggression* (IND); (d) *verbal latent aggression* (VL), and (e) *physical latent aggression* (PHL). The subject's answers were given on a five-point scale: (1) they never behave in that way, (2) they behave seldom in that way, (3) they behave in that way from time to time, (4) they behave frequently in that way, and (5) they

behave very often in that way. Total test score may lie in the range between 75 and 375 points. Research has shown that the A-87 has satisfactory psychometric properties [27–29].

2.2.5. Beck Anxiety Scale. The Beck Anxiety Inventory (BAI), created by Dr. Aaron T. Beck and other colleagues, is a 21-question multiple-choice self-report inventory that is used for measuring the severity of an individual's anxiety. The BAI consists of twenty-one questions about how the subject has been feeling in the last week, expressed as common symptoms of anxiety (such as numbness, hot and cold sweats, or feelings of dread). Each question has the same set of four possible answer choices, which are arranged in columns and are answered by marking the appropriate one with a cross. These are (1) not at all, (2) mildly: it did not bother me much, (3) moderately: it was very unpleasant, but I could stand it, (4) severely: I could barely stand it. In our study the version of this test with 14-question multiple-choice self-report inventory was used [30, 31].

2.3. Laboratory Testing. Blood samples were analyzed for blood glucose levels and plasma catecholamine levels at the Department of Clinical Laboratory Diagnostics, University Hospital Center Osijek. Adrenaline and noradrenalin levels were evaluated by using commercially available reagent kit (Catecholamines in plasma, Chromsystems, Germany) which allows the routine analysis of adrenaline and noradrenalin in plasma using an isocratic high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) and an electrochemical detector (ECD, Chromsystems, Germany).

2.4. Statistical Analysis. All results are presented as mean ± SD. The normality of data distribution was assessed by Kolmogorov-Smirnov Normality test. Clinical characteristic between two measurements (before and after Red Bull© consumption) were compared by paired t-test. Wilcoxon rank-sum test was used when variables were not normally distributed. To compare parameters between experimental protocols Student's t-test was used. When variables were not normally distributed Mann Whitney Rank Sum Test was used. To compare differences between catecholamine levels during different time periods One-Way ANOVA Repeated Measures was used. Statistical significance was set at $P < 0.05$. For statistical analysis Sigma Plot (version 11.2, Systat Software, Inc., Chicago, USA) program was used.

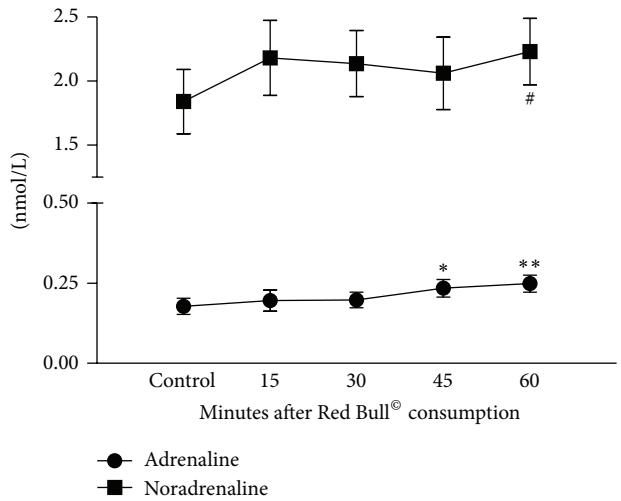
3. Results

Thirty-eight young healthy college students with a mean age of 23 ± 2 years completed the study (15 female and 23 male participants). BMI of 23.95 ± 3.03 kg/m^2 and WHR of 0.79 ± 0.05 were measured. Mean of three BP measurements confirmed normotension before Red Bull© consumption among participants. Table 1 summarizes the values of arterial BP and HR of study population. Systolic blood pressure (SBP) was not significantly changed after Red Bull© intake. However, diastolic blood pressure (DBP) was significantly increased after Red Bull© consumption, compared to control measurement. Mean arterial pressure (MAP) was significantly increased 30 minutes after Red Bull© consumption. There was significant increase in heart rate 30 minutes after Red Bull© intake, too. All participants had normal fasting blood glucose level with no history of impaired glucose intolerance or diabetes mellitus. Blood glucose level significantly rose 30 minutes after Red Bull© consumption (plasma glucose mmol/L before Red Bull© consumption 4.6 ± 0.4 versus after Red Bull© consumption 7.0 ± 1.4, $P < 0.001$).

Table 1: Arterial blood pressure and heart rate of study population.

Variable	Before Red Bull©	After Red Bull©	P value
SBP	113 ± 12	115 ± 12	0.151
DBP	68 ± 10	70 ± 8*	**0.03**
MAP	83 ± 10	85 ± 9*	**0.037**
HR	71 ± 10	76 ± 11*	**<0.001**

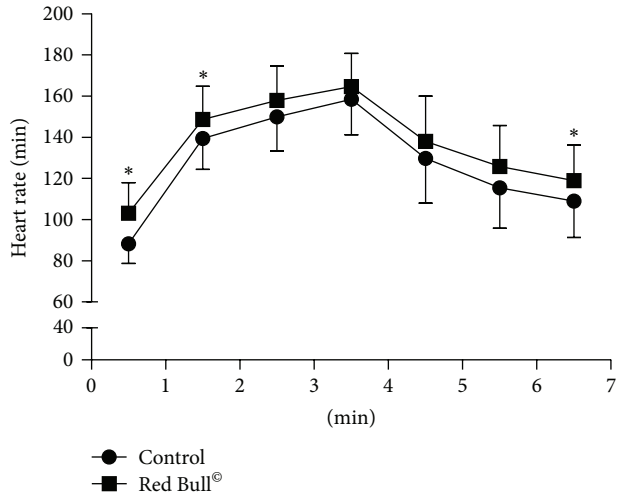
Results are expressed as mean ± SD.
SBP: systolic blood pressure, DBP: diastolic blood pressure.
MAP: mean arterial pressure, HR: heart rate.
*$P < 0.05$ (statistically significant values are in bold) before Red Bull© versus after Red Bull© ($n = 38$).



Data are expressed as mean ± SD
*$P < 0.05$, **$P < 0.01$ plasma adrenaline levels control versus after Red Bull© ($N = 9$, number of subjects measured before and after Red Bull© consumption)
#$P < 0.05$ plasma adrenaline levels control versus after Red Bull© ($N = 9$, number of subjects measured before and after Red Bull© consumption)

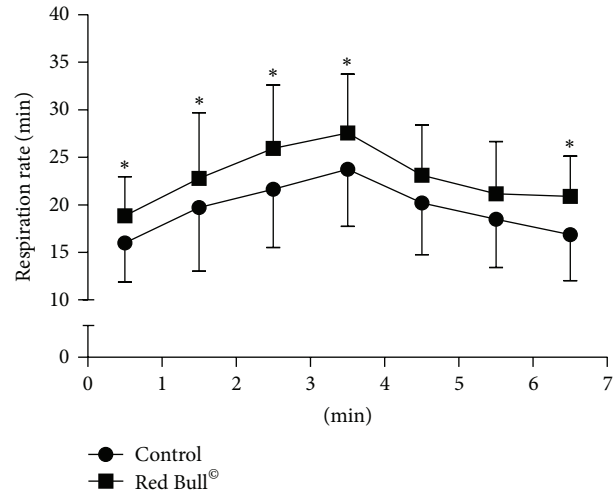
Figure 1: Influence of Red Bull© on plasma adrenaline and noradrenalin levels.

3.1. Influence of Red Bull© Consumption on Plasma Adrenaline and Noradrenalin Levels. Figure 1 summarizes influence of Red Bull© consumption on plasma adrenaline and noradrenalin levels that were measured in five different time points (before Red Bull© consumption and 15, 30, 45, and 60 minutes after Red Bull© consumption). Both adrenaline and noradrenalin plasma levels tended to increase after Red Bull© consumption in all measured time points with statistical significance for adrenaline ($P = 0.0096$) at 45 and 60 minutes time points compared to before Red Bull© consumption



Data are expressed as mean ± SD
* $P < 0.05$ control versus Red Bull© ($N = 29$, number of subjects measured before and after Red Bull© consumption)

Figure 2: Change of heart rate (HR) during 1-minute rest, 3 minute of Harvard Step Test, and 3-minute rest after exercise before and after Red Bull© consumption.



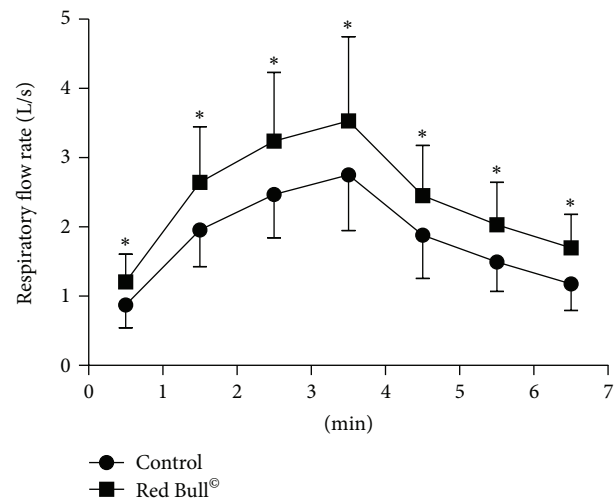
Data are expressed as mean ± SD
* $P < 0.05$ control versus Red Bull© ($N = 29$, number of subjects measured before and after Red Bull© consumption)

Figure 3: Change of respiration rate (RR) during 1 minute rest, 3 minute of Harvard Step Test and 3 minute rest after exercise before and after Red Bull© consumption.

and noradrenalin ($P < 0.0001$) at 60 minutes time points compared to before Red Bull© consumption.

3.2. Influence of Red Bull© Consumption on Cardiac and Respiratory Changes during Harvard Step Test. Measurement of HR, respiration rate (RR), and respiratory flow rate (RFR) during 1-minute rest before the beginning of Harvard Step Tests (HST) has shown significant changes after Red Bull© consumption. The first-minute HR during rest before the beginning of HST was significantly increased after Red Bull© intake compared to control condition before Red Bull© intake (HR/min 89 ± 10, 103 ± 14, resp.; $P < 0.001$). In the same condition, RR and RFR were also significantly increased after Red Bull© consumption compared to control condition before Red Bull© intake (RR/min 16.0 ± 4.1, 18.8 ± 4.1, $P < 0.001$; RFR L/s 0.87 ± 0.33, 1.21 ± 0.4, resp.; $P < 0.001$) (Figures 2, 3, and 4). Skin temperature (ST) was not significantly changed during rest after Red Bull© intake, compared to control resting condition before Red Bull© intake (ST °C 32.1 ± 0.3, 32.2 ± 0.4, resp.; $P = 0.081$).

Same parameters were measured during 3 minutes of HST before and 30 minutes after Red Bull© intake. As expected, HR, RR, and RFR were increasing with the exercise duration before and after Red Bull© consumption. ST did not change during 3 minutes of HST before and after Red Bull© intake. HR was significantly higher ($P < 0.001$) during first minute of HST after Red Bull© consumption compared to the control (Figure 2). RR and RFR were statistically significantly higher during all 3 minutes of HST after Red Bull© intake compared to the control HST (Figures 3 and 4). ST did not change during exercise after Red Bull© compared to the control HST.



Data are expressed as mean ± SD
* $P < 0.05$ control versus Red Bull© ($N = 29$, number of subjects measured before and after Red Bull© consumption)

Figure 4: Change of respiratory flow rate (RFR) during 1-minute rest, 3-minute of Harvard Step Test, and 3-minute rest after exercise before and after Red Bull© consumption.

As expected, HR, RR, and RFR were decreasing during 3 minutes rest after control HST and HST that followed Red Bull© intake. Still, HR (Figure 2) and RR (Figure 3) were significantly higher during third minute at the rest after HST following Red Bull© intake compared to control before Red Bull© consumption (Figure 2). RFR was significantly higher during all 3 minutes at the rest after HST following Red Bull© consumption, compared to the control (Figure 4). ST was increasing during all three minutes of rest after pre-Red Bull©

Table 2: Scores achieved on tested cognitive tasks.

Cognitive function	Task	Before Red Bull©	After Red Bull©	*P* value
Immediate memory	AVLT- (1-) first trial	8,17 ± 1,77	7,79 ± 2,04	0,426
	ADST-forward	8,03 ± 0,82	8,48 ± 0,87*	**0,029**
Delayed recall	AVLT-delayed recall	12,58 ± 2,18	13,00 ± 1,93	0,231
Working memory	ADST-backwards	6,45 ± 0,83	6,86 ± 1,03*	**0,048**
Delayed verbal memory	AVLT-late recall	12,55 ± 1,97	12,03 ± 3,15	0,459
Verbal fluency	Semantic task	23,28 ± 3,89	26,10 ± 4,94*	**<0,001**
	Ideational task	15,07 ± 4,00	18,66 ± 4,43*	**<0,001**
	Phonemic task	30,70 ± 7,31	41,76 ± 9,71*	**<0,001**
Attention	d2 test TN-E	486,83 ± 69,19	592,45 ± 60,31*	**<0,001**
	d2 test MC	267,90 ± 27,90	283,21 ± 21,89*	**<0,001**

Results are expressed as mean ± SD.
AVLT: auditory verbal learning test; ADST: auditory digit span task; TN-E: quantitative measure defined by total number of processed data minus number of total errors; MC: mean concentration.
* $P < 0.05$ (statistically significant values are in bold) before Red Bull© versus after Red Bull© ($n = 27$).

control HST and HST following Red Bull© intake, but without differences between these two measurements.

3.3. Influence of Red Bull© Consumption on Reaction Time. Reaction time (RT) was measured before and after Red Bull© consumption. In both measurements, subjects reacted faster when sound signals appeared in randomized time intervals compared to the RT when sound signals appeared in fixed time intervals (RT sec before Red Bull© 0.26 ± 0.04, 0.29 ± 0.04, $P = 0.002$; after Red Bull© 0.24 ± 0.04, 0.27 ± 0.03, $P < 0.001$). Subjects had faster RT after Red Bull© consumption compared to the control pre-consumption condition in both test, independently on sound signals appearing in fixed ($P < 0.001$) and randomized ($P < 0.001$) time intervals.

3.4. Influence of Red Bull© Consumption on Cognitive Functions. Table 2 presents the results achieved in particular cognitive tasks. Results achieved in immediate memory (measured as number of correctly recalled words after first reading) in AVLT did not show any difference after Red Bull© consumption compared to control. However, immediate memory (measured as number of correctly repeated numbers in forward order) in ADST was significantly improved after Red Bull© intake compared to control. There was no significant difference in delayed recall or verbal memory measured by AVLT in control test compared to test after Red Bull© intake. Working memory (measured as number of correctly repeated numbers in backward order) was significantly better after Red Bull© intake. Verbal fluency (phonemic, semantic, or ideational) was significantly better after Red Bull© consumption compared to control measurement. There was a significantly better performance in the d2 attention loading task (TN-E and MC) after Red Bull© intake compared to the performance in control d2 attention loading task.

3.5. Influence of Red Bull© Consumption on Emotional Status and Mental Stress Response. Participants' response to the mental stress test (MST) was significantly better after Red Bull© intake compared to the control (MST points control 146.56 ± 50.73 versus points after Red Bull© 189.31 ± 48.03, $P < 0.001$). However, subjects performance in Aggression Questionnaire AG-87 (AG-87 0 participants had same answers before and after Red Bull© consumption, 16 participants (55.17%) had more points before Red Bull© consumption and 13 participants (44.83%) had more points after Red Bull© consumption; $P = 0.502$) and Beck Anxiety Inventory did not show any significant differences between two measurements, before and after Red Bull© consumption (BAI 23 participants (79.13%) had same answers before and after Red Bull© consumption, 4 participants (13.79%) had more points before Red Bull© consumption, and 2 participants (6.9%) had more points after Red Bull© consumption; $P = 0.414$).

4. Discussion

The salient finding of the present study is that Red Bull© consumption (1) increased activity of the sympathetic nervous system (by increased plasma adrenaline and noradrenalin levels); (2) subsequently affected cardiovascular and respiratory system during rest and exercise; and (3) improved cognitive functions, as well as the performance in mental stress test and reaction time, while (4) Red Bull© consumption did not have significant effect on subject's emotional status, precisely aggression and anxiety. Red Bull© consumption significantly increased MAP and HR as well as RR and RFR, at rest and during exercise in young healthy population, all of which are under adrenergic control; it had beneficial effect on cognitive functions (immediate memory, attention, and verbal fluency), improved performance in MST, and improved RT. However, consumption of Red Bull© has not altered subject's emotional status, including aggression and anxiety. 500 mL Red Bull© consumption significantly increased blood glucose levels, as well as plasma adrenaline and noradrenalin levels. To our knowledge this is the first study which demonstrated that Red Bull© consumption leads to adrenergic system activation with subsequent changes in both cardiovascular and psychomotoric reactions in young individuals.

We have found significantly increased DBP and MAP while SBP increased but without significance after Red Bull© consumption. HR was significantly increased 30 minutes

after Red Bull© intake. These results are consistent with Steinke et al. who observed influence of 500 mL energy drink through 7 days and reported elevated DBP within 2 hours of energy drink consumption as well as elevated SBP and HR at day 1 of energy drink consumption [8]. A novel study from 2014 has shown that Red Bull© consumption led to increases in both SBP and DBP, associated with increased HR and cardiac output, with no significant changes in total peripheral resistance and without diminished endothelial response to acetylcholine [32]. Even though some authors reported decreased HR after consuming energy drink, we found opposite changes in concordance to [4, 7, 9]. Inconsistency of the results that described the BP changes are probably caused by quantity of energy drink used in the study as well as a fasting period for caffeine before study protocol [7]. It was reported that consumption of a single can of Red Bull© (250 mL) was not associated with adverse cardiovascular effects [33]. Repeated HR measurement during one-minute rest before HST, confirmed increased HR after Red Bull© consumption compared to control measurement. HR was significantly increased during the first minute of exercise, as well as during the third minute of rest after exercise. This increment of arterial blood pressure and HR 30 minutes after Red Bull© consumption may be associated particularly with increased sympathetic activity. Today is generally accepted that sympathetic overactivity results in increased HR and blood pressure [34, 35]. We cannot be sure which substances in Red Bull© are responsible for this effect, but caffeine is a likely candidate. Although it is tempting to attribute the BP- and HR-elevating effects of Red Bull© to its caffeine content and consequent sympathetic adrenal-medullar system activation, a recent pilot study [36] reported that repeated consumption of Red Bull© drinks between 8:00 and 19:00 led to an increase in mean 24 h and daytime ambulatory BP when compared to caffeine consumption alone. This raises the possibility that other ingredients in Red Bull©, in their own rights or in interaction with caffeine, may underline elevation in BP and HR after Red Bull© consumption only by adrenergic system activation and/or by involvement of another mechanism which still remains to be determined. In order to clarify this issue, it would be very important to test whether this BP- and HR-elevating effect of Red Bull© would be diminished by the use of adrenergic antagonists in some future studies.

Blood glucose level significantly rose 30 minutes after Red Bull© consumption. This significant increment can be attributed to intake of almost 56 g of sugars (glucose 10,5 g and saccharose 43 g) in 500 mL of Red Bull©. However, it has been shown that activation of adrenergic system via physical or emotional stress increases blood glucose levels (max glucose levels reached 30 minutes after stress stimulation) [37]. Furthermore, the effects of caffeine, particularly on glucose metabolism in the sedentary state are detrimental. Acute consumption of caffeine in combination with a glucose load is known to transiently impair whole-body glucose disposal and to cause hyperinsulinemia as well as hyperlipidemia. Caffeine interferes with the actions of insulin, and it is important to appreciate that this hormone affects the metabolism of lipids as well as carbohydrates [38]. With regard to carbohydrate homeostasis, caffeine containing energy drinks deliver both a readily absorbable form of glucose in combination with a dose of caffeine sufficient to impair glucose metabolism. Thus the acute and chronic influence of caffeine and sugars containing energy drinks which, according to our results, activate adrenergic system on blood glucose levels, require further careful consideration in future studies.

To our knowledge, this is the first study that measured the impact of Red Bull© consumption on respiratory function at rest and during exercise. We found significantly increased RR as well as RFR at rest, during exercise and at rest after exercise. This effect of Red Bull© can also be explained by activation of sympathetic nervous system, which leads to increased RT and greater alveolar oxygen exchange due to bronchioles dilation.

However, although potential link between energy drink consumption and increased sympathetic activity is accentuated in numerous studies, there is a lack of actual measurement of plasma catecholamine levels in response to Red Bull© to support these speculations. That is the reason why in the present study we have measured plasma concentrations of adrenaline and noradrenalin in five different time points (before Red Bull© consumption and 15, 30, 45 and 60 minutes after Red Bull© consumption) and demonstrated that both adrenaline and noradrenalin plasma levels were increased after Red Bull© intake through the time in which different study protocols were randomly performed compared to values before Red Bull© intake (Figure 1). This is firm evidence that indeed sympathoadrenal activation is underlying mechanisms of cardiovascular and respiratory effects of RedBull©. As mentioned above, final confirmation of this connection between Red Bull© consumption and activation of adrenergic system, would be diminished cardiovascular and respiratory effect of Red Bull© consumption by adrenergic antagonist which is the natural next step in further studies.

Our finding that Red Bull© intake improved RT is consistent with Alford et al. who reported that Red Bull© improved reaction times, alertness and concentration, mental acuity skills that might be considered important to college students [7]. Seidl et al. have found significantly improved motor reaction after administration of caffeine-and-taurine-containing drink, similar to Red Bull© [19].

As Red Bull© is advertised and sold as energy drink that beside physical, improves and mental performance, it is really important to find its real effect on cognitive functions. Results of our study have shown significant improvement in working memory, verbal fluency and attention tests performance after Red Bull© consumption. Immediate memory was significantly improved after consuming Red Bull©, when measured as number of correctly repeated numbers in forward order. In already mentioned study, Alford et al. found that Red Bull© improved cognitive performance [7]. The results of Howard and Marczinski indicated that the energy drink doses decreased RTs on the behavioral control task, increased subjective ratings of stimulation and ratings of mental fatigue, suggesting improved cognitive performance [20]. Seidl et al. reported improved attention, measured with same test as in our study (d2 Attention Loading Test), after administration of caffeine-and-taurine-containing drink, similar to Red Bull© [19]. Although most studies that examined the influence of energy drink on cognitive performance

have shown positive effect, mechanisms are still not clear. Mechanisms for modulation of cognitive functions by the energy drink can be assigned to caffeine via its action on adrenosinergic system, which in turn is closely linked to other neurotransmitter systems [39]. Important finding in our study is a better success in MST after Red Bull© consumption. Some previous studies have reported similar effect, which is contributed to the taurine, known to modulate mood as well as stress and behavioral response [40–42]. Interestingly, even though it is known that catecholamines appear to be involved in metabolic preparations for the prospective fight and that a slight activation of noradrenergic system stimulate aggression, we did not observe significant effect of Red Bull© consumption with subsequent increased activity of the sympathetic nervous system on measured impulsive aggression [43, 44]. Also, we did not observe significant effect of Red Bull© consumption on individual's anxiety although numerous studies have described increased sympathetic activity in patients with depression and anxiety [45], which is important in the light of increased aggressive behavior among young people. However, it should be emphasized that there has been a dramatic rise in the consumption of alcohol mixed with energy drinks in young people which has been implicated in risky drinking practices, greater accidents and injuries, and risky behavior like risky sexual behavior and violence [46, 47]. Since it is currently not entirely clarified how the combined effect of alcohol and energy drinks impact the activation and inhibition of behavior differently than alcohol would alone, more controlled laboratory studies are needed to determine if alcohol mixed with energy drinks are escalating risky drinking practices among young people.

Limitations of the study: the study was organized as before and after the treatment study, where subjects were self-controls. Due to particular taste of Red Bull©, it was not possible to blind subjects or examiners on the beverage taken. Because Red Bull© intake obviously has important effect on glucose metabolism and blood glucose level, a glucose tolerance test would be interesting to perform, to exclude possibility that glucose is high due to intolerance and not due to high glucose content in the Red Bull© to. However, as stated in Methods section, participants in this study were healthy young people with normal fasting glucose levels and no positive history of diabetes or prediabetes or glucose intolerance.

In conclusion, our study has shown consistent positive effects of Red Bull© energy drink on cognitive functions (immediate memory, attention, and verbal fluency), improved performance in mental stress test and improved reaction time. These positive effects are consistent with the assertions of Red Bull© manufacturers and one of the main reasons why are precisely young people and college students the main consumers of Red Bull©. However, results of this study have shown that 500 mL of Red Bull© may have adverse effect on cardiovascular and respiratory system, leading to increased MAP and HR as well as RR and RFR, at rest and during exercise. Precisely because the target market for Red Bull© is people between 15 and 30 years of age, that is, typically healthy, involved in activities, and includes a higher proportion of sports enthusiasts and high-risk takers, these effects should not be neglected. Other main problems involved in Red Bull© consumption are that its numerous ingredients and its effect is difficult to assign to one specific ingredient. This is first study that has actually shown increased activity of the sympathetic nervous system (increased plasma adrenaline and noradrenalin levels) after Red Bull© consumption which may have an important role in its observed effects on cardiovascular and respiratory system during rest and exercise, on cognitive functions as well as on performance in mental stress test and reaction time and thus elucidated important mechanism of its' action.

Ethical Approval

The study protocol and procedures conformed to the standards set by the latest revision of the *Declaration of Helsinki* and were approved by the Ethical Committee of Faculty of Medicine, Josip Juraj Strossmayer, University of Osijek.

Consent

Written informed consent was obtained from each subject.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Ana Cavka and Marko Stupin equally contributed to this paper.

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Research Article

Myocardial Infarction and Stroke Risk in Young Healthy Men Treated with Injectable Testosterone

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This study was conducted to examine the association between testosterone therapy and new myocardial infarction (MI) and stroke events in a series of patients treated at Low T Centers across the United States, consisting of mainly young (mean age = 46), otherwise, healthy men. Electronic medical records were queried between the years 2009 and 2014 to identify patients diagnosed with hypogonadism, MI, and stroke, as indicated by ICD-9 codes. The incidence of MI and stroke events was compared to community-based registries. 39,936 patients recruited from 40 Low T Centers across the United States were treated and 19,968 met eligibility criteria for receiving testosterone treatment. The incidence rate ratio (IRR) for MI in testosterone- (T-) treated versus nontreated patients was 0.14 (C.I. = 0.08 to 0.18, $P < 0.0001$) whereas the IRR for stroke for T-treated versus nontreated patients was 0.11 (C.I. = 0.02 to 0.13, $P < 0.0001$). There was no evidence of worsening preexisting MI or stroke in patients treated with testosterone. The experience in Low T Centers shows that, in an injectable testosterone patient registry, testosterone is generally safe for younger men who do not have significant risk factors. Of patients that developed MI with testosterone, there was no association with testosterone or hematocrit levels.

1. Introduction

In the past year, concerns have been raised over the safety of testosterone replacement therapy (TRT) because of two peer-review papers associating myocardial infarctions (MI) and strokes with TRT use by men [1, 2]. These studies have been followed by a flurry of potential litigation against manufacturers of testosterone. In addition, great confusion has arisen for both patients and their treating physicians.

In the first article by Vigen et al. [2], the authors initially excluded 1132 men from analysis who had received a testosterone prescription after experiencing an event (MI or stroke) but, later, published an erratum in 2014 disclosing that the number of patients excluded should have been 128, not 1132, resulting in an 89% error rate. Among the original group of 1132 excluded individuals, 100 patients were in fact women, not men. Moreover, an additional exclusion criterion based on either missing coronary anatomy data or data classification as "other" was incorrect and changed from 1301 to 397 patients. Despite strong media attention on these findings, these clear inconsistencies in data reporting undermine the credibility of these findings.

In the 2014, article by Finkle et al. [1], the authors compared nonfatal MI among men prescribed testosterone versus PDE5 inhibitors (PDE5Is) for treatment of hypogonadism. A critical limitation of this study was that testosterone levels of men prescribed PDEs were unknown, either at baseline or after treatment. The study relied on insurance data; patients were on variable treatment protocols not defined in the study. The authors compared a group of men with presumably low testosterone who may not have received adequate treatment for hypogonadism against an unrelated cohort of men with unknown but presumed average testosterone levels. Therefore, two treatment groups were not comparable and the interpretation of the study was limited.

The release of these two peer-review articles prompted the US Low T Center to initiate an internal quality management program to determine whether its patients were adversely impacted by higher risk of MI or stroke following initiation of testosterone treatment. The Low T Centers are a privately owned group of 50 clinics distributed across the United States [3]. These clinics are staffed by board certified physicians in various specialties as well as physician assistants. The protocols for determining treatment are specific to Low T Centers, modified from established guidelines from both the Endocrine Society and the American Association of Clinical Endocrinologists [4, 5]. Patients are selected for treatment if deemed hypogonadal, defined as total testosterone <350 ng/dL or free testosterone <10 ng/mL. In addition, patients cannot have contraindications such as prostate cancer, breast cancer, polycythemia, severe obstructive sleep apnea, and/or severe untreated lower urinary tract symptoms (LUTS). Approximately, 19,968 of 40,000 (50%) patients that seek treatment in these Low T Centers do not qualify after screening tests. Among those who do qualify, they must undergo supervised, short acting injection treatments, requiring clinic visits every one to two weeks. During these visits, additional clinical parameters such as blood pressure, testosterone, and estradiol levels are closely monitored.

This study consists of an examination of the incidence of myocardial infarction (MI) and/or stroke in a large, multicenter practice (Low T Centers) with uniformity in treatment protocols and adherence to standards of care due to concerns for the safety of patients taking testosterone within such large, multisite practices.

2. Subjects and Methods

This study consisted of a retrospective, multicenter medical chart review across 40 participating US Low T Centers, with a geographical concentration in Texas. At these centers, patients received mainly intramuscular testosterone cypionate once weekly or every 2 weeks. Blood draw was every 90 days but may be earlier based on clinical indications (Figures 1 and 2). Prior to systematic data extraction, three investigator conference calls were held with Low T Center providers to ensure that all International Center for Diseases-(ICD-) 9 codes were updated, with particular attention to MI and stroke. Investigators also interviewed families with patients that had sudden deaths, presumed to be fatal MI. The principal investigator at each site also underwent training with the information technology specialist at his/her respective Low T Center, including training on accessioning the patient's electronic health record (EHR). Researchers also underwent Good Clinical Practice (GCP) training with the Food and Drug Administration. Approval was obtained from the Aspire Institutional Review Board (IRB) to conduct the study using EHR chart reviews. Each research site captured deidentified data on case report forms using numeric codes to ensure patient confidentiality.

The medical records of all male patients evaluated in the Low T Centers between October 1, 2009, and March 15, 2014, were queried for a diagnosis of hypogonadism, myocardial infarction, and stroke using the following ICD-9 codes: 257.2, 412, 410.80, 438.89, and 436. In order to be diagnosed with hypogonadism, the patient was required to have reported symptoms in addition to having had a serum testosterone level below 350 ng/dL. This cut-off was determined by the protocol committee at Low T Centers. Records for patients initiating testosterone replacement therapy (TRT) and having blood testing for hormone assays were reviewed. Inclusion in the study required availability of full demographic information, male gender, age ≥ 20 years, and a history of at least one visit to a Low T Center, whether treatment was received or not. All data were recorded in an Excel spreadsheet with patient information linked to a unique identifier to maintain patient confidentiality. For baseline and follow-up visits, the visit date, ICD-9 codes, and adverse events for those on TRT were recorded from the ICD-9 diagnoses available in the EHR system "Advance MD" [6]. Of the patients having a record of MI or stroke, the practitioners were advised to contact the patient for further interview. Total testosterone levels were drawn in the clinic and results were given to the patient within 30 minutes. Testosterone assays were performed primarily by the Qualigen FastPack IP TestoImmunoassay, which is a chemiluminescent immunoassay for the in vitro quantitative determination of total testosterone in human serum. Estradiol levels were measured using electrochemiluminescence immunoassay (ECLIA) through Lab Corp. Free testosterone levels were calculated from total testosterone and sex hormone binding globulin (SHBG).

The statistical significance of between-cohort differences in categorical variables was tested using the chi-square test and for continuous variables using the two-sample Student's t-test. All tests were two-tailed with a significance level of $P < 0.05$. Analyses were conducted using SPSS and SYSTAT.

This study focused on five comparison registries for MI incidence [7–10]. As patients in Low T Centers represented primarily commercial health insurance patients, the Kaiser Permanente database in Northern California (which was also mainly commercial insurance) was chosen for comparison. Since Low T Centers are distributed across the US, an additional 4 registries were included for comparison from different parts of the country. No single registry with a predominantly younger population could be identified. The average rate of MI from the other four combined US-based registries was 203 events per 100,000 persons which approximates that of the Kaiser site at 208 events per 100,000 persons. Rates of MI varied from 71.6 to 241 events per 100,000 persons. The Northern Manhattan Registry data were selected for comparison of stroke events because of the known rigor in data collection and quality [11].

3. Results

3.1. Patient Population. Data were extracted from the electronic health records (EHR) of the 40 medical centers across the United States. All subjects were male and the age range was 20 to 86 years, with 87% of patients being less than 55 years old and only 3% being greater than 65 years old. From a total population of roughly 40,000 patients, this study contributed to up to 160,000 person-years of observation.

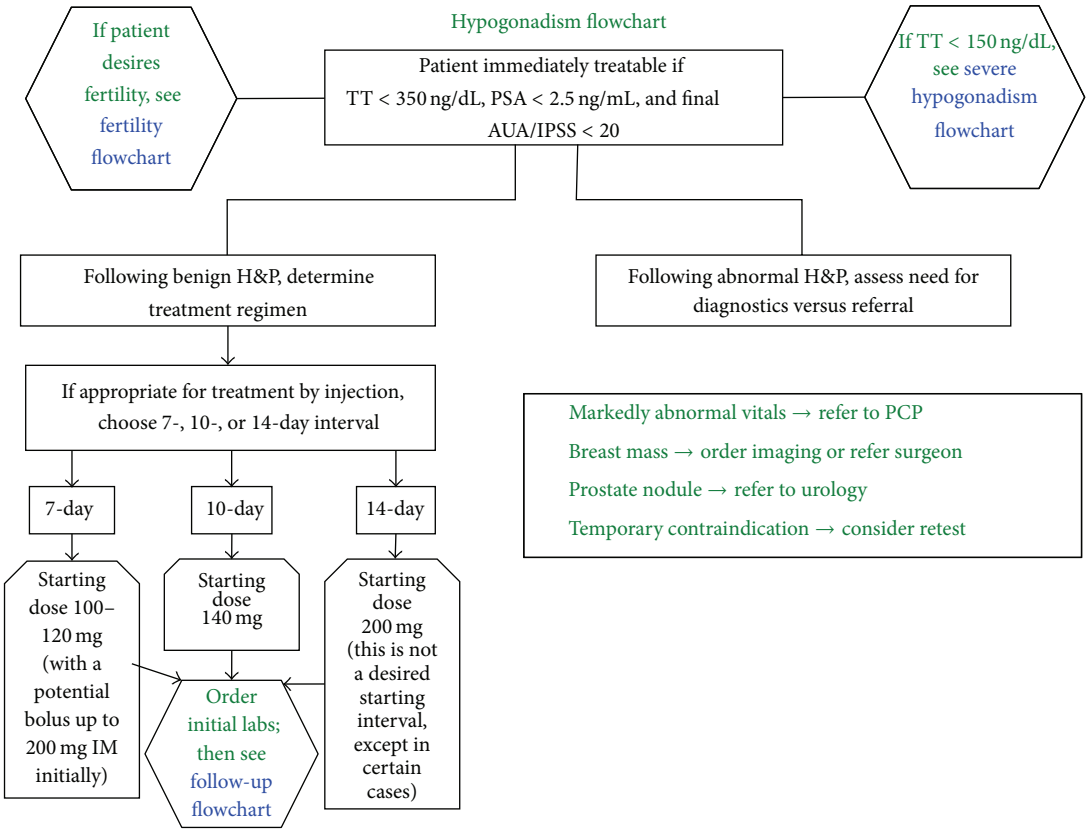


Figure 1: Flow diagram for patient enrolment protocol in a Low T Center.

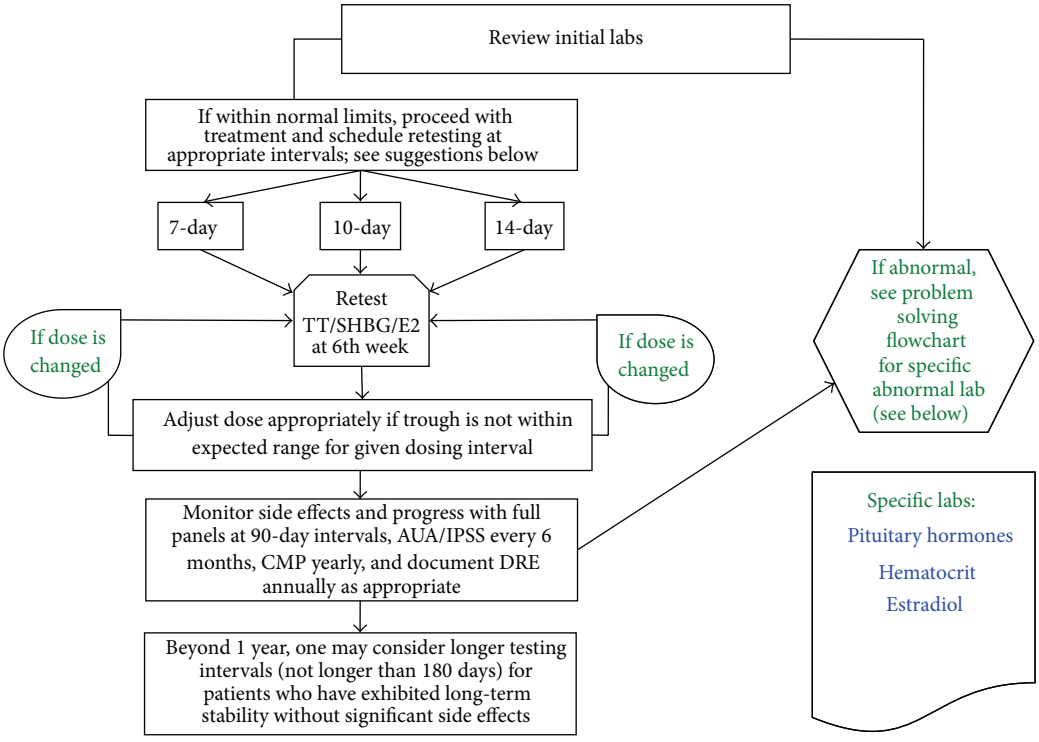
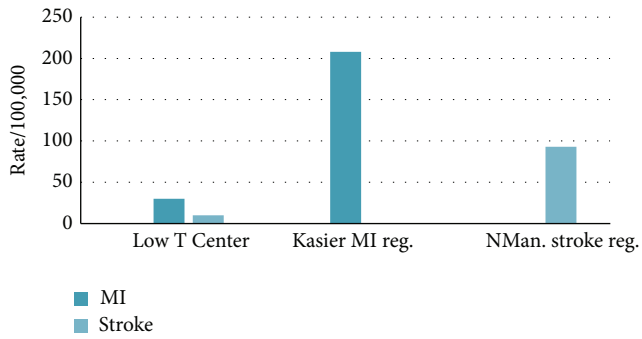


Figure 2: Flow diagram for follow up patients at a Low T Center.

Table 1: Incidence rate of myocardial infarction (MI) from various registries (per 100,000 persons).

Study	Rate	Comments
United States National Hospital Discharge Survey, 2002	242	26-year study and noted case fatality rates decreased over time
New York State Registry (1996–2008)	71.6	13-year study and noted decrease mortality with time
Marshfield, Wisconsin, Epidemiology Study, 2002	292.4	6-year study of MI rates in stable population in WI
Fukushima Prefecture, Japan, 2013	37.9	Rates of MI were compared before & after the Tsunami
Kaiser Permanente, Northern California, 2008	208	The average of rates of MI from the 4 US registries approximates that of Kaiser at 203 per 100,000
Low T Centers, United States, 2014	30.0	Patients received testosterone injections



	Low T Centers	Selected community registries	Incidence rate ratio (IRR)*	Confidence interval, P value
Myocardial infarction	30/19,968 treated patients	208/821,725 hospitalizations (Kaiser)	0.14	0.08 to 0.18, $P < 0.0001$
Stroke	10/19,968 treated patients	93/117,000 residents (NMan.)	0.107	0.02 to 0.13, $P < 0.0001$

*IRR is defined as relative difference measure used to compare incidence rates of events.

Figure 3: Summary of cardiac events in Low Testosterone (T) Centers and comparison registries (per 100,000 persons).

The characteristics of the study population, as well as the comparable registries, are summarized in Table 2. Anthropometric measurements were not completed in some Low T Centers and, as such, not reported in this publication.

3.2. Treatment Outcomes. The rate of MI and stroke varied across the country. A summary of these rates is presented in Table 1. Between years 2009 and 2014, 39,937 patients were seen and approximately 50% met criteria for treatment. Of the treated patients, there were 4 nonfatal MI and 2 probable fatal MI; thus the rate of new MI was 30 events per 100,000 persons. The 2 probable cases were presumed to be from MI because of sudden death; since no postmortems were performed and the 2 deaths could have been from other causes, they were still included in the analysis. There were 46 patients with pretherapy MI of which none had adverse outcomes after testosterone. Of the treated patients, there were two cases of stroke; thus, the rate of new strokes was 10 events per 100,000 persons. There were 12 patients with pretherapy stroke and none had adverse outcomes after testosterone. The risks for new MI and stroke were compared to the Kaiser Permanente and Northern Manhattan Registry which were 208 events per 100,000 persons and 93 events per 100,000 persons, respectively. As the treated population base at Low T Centers was 19,968 patients and, to match the controls, the rate was given a multiple of 5 times, thus, the incidence rate ratio (IRR) for MI in testosterone-treated patients is 0.14 (C.I.: 0.08 to 0.18, $P < 0.0001$) whereas strokes is 0.11 (C.I.: 0.02 to 0.13, $P < 0.0001$) (Figure 3).

Overall, the mean time of exposure to testosterone for all patients in our study was 17 months. Among those with cardiovascular events, there appears to be no association of time of MI or stroke with the duration of exposure to testosterone. MI and stroke occurred at various times of exposure, ranging from 4 weeks to 4 years. In a particular case of a 55-year-old patient, the cardiac event occurred after 6 weeks of treatment, with the observation that both testosterone and estradiol rose 3 times from baseline. The association of high estradiol from aromatization from testosterone to the cardiac event is likely serendipitous; but there will be further analysis of this data, which will be beyond the boundaries of this publication. The results are summarized in Table 3.

The mean total testosterone levels of all patients were determined. For patients aged 25 and younger, the level was 432 ng/dL; for patients aged 25–44 years, the level was 460 ng/dL; for patients aged 45–65, the level was 503 ng/dL;

Table 2: Characteristics of Low Testosterone (T) Center patients in comparison to controls from other registry populations.

	Low T Centers	Kaiser Permanente	Northern Manhattan Registry	Comments
Male (%)	100	62	45	
<55 years (%)	87	Not reported	74	Kaiser reported age as 69 ± 14
>56 years (%)	13	Not reported	26	3% Low T > 65
White (%)	Not reported	67	22	Low T Centers did not collect ethnicity data
Black (%)	Not reported	7	13	
Hispanic (%)	Not reported	9	64	
Asian and others (%)	Not reported	17	1	
Hypertension (%)	15	76	Not reported	N Manhattan registry has high percentage of minorities which will imply higher rates of HTN
DM (%)	4	32	Not reported	N Manhattan registry has high percentage of minorities which will imply higher rates of DM
Hyperlipidemia (%)	12	80	Not reported	N Manhattan registry has high percentage of minorities which will imply higher rates of HLD

and, for patients above 65 years, the level was 552 ng/dL (data not shown). Measurements of the mean total testosterone level could occur at the beginning, middle, or end of the injection cycle. As such, the range of total testosterone varied from 50 to 1600 ng/dL. However, the mean testosterone level of all treated patients irrespective of age was 543 ng/dL.

The Low T Centers see patients that are generally much younger than in a typical urological or endocrinology practice. The mean initial total testosterone level of those that received testosterone treatment and who were below 25 years was 178 ng/dL. The typical patient is often self-referred rather than being referred from another physician. Also, the approach may be more preventive. Of the patients seen at the Low T Centers, the prevalence rates of hypertension, diabetes, and hyperlipidemia were 15%, 4%, and 12%, respectively. Comorbidities were reported by the patient and confirmed by the treating provider and entered in the problem list. The population seen at the Low T Centers contrast from a typical academic andrology practice, where there may be a higher prevalence of comorbidities [12].

The levels of testosterone were studied in patients with MI. Of the patients that had MI, the last mean total testosterone was 480.50 ng/dL and was lower than the mean total testosterone of all treated patients, which was 543.0 ng/dL.

4. Discussion

In the past, most of the literature supported the role of endogenous testosterone in protecting the cardiovascular system [13, 14]. In some small interventional studies, testosterone has been shown to have vasodilatory properties which implied cardioprotection [15]. In epidemiological studies, elevated blood pressure is an established risk factor for heart attacks and strokes. Blood pressure was found to be inversely correlated to testosterone levels [16]. Studies also show that testosterone suppression leads to accelerated atherosclerosis [17].

However, two recent papers found an increased risk of MI and strokes [1, 2]. This caused widespread concerns among practitioners who prescribe testosterone use. In fact, practitioners were so concerned that a group of experts formed the Androgen Study Group to address the findings of these studies [18]. Moreover, several important challenges to these findings have since been published [19–21].

In this study, it was observed that the rates of MI and stroke in a multicenter practice were very low, in contrast with these two recent papers [1, 2]. Instead of associating TRT with MI and stroke, the Low T Center data paradoxically demonstrated that TRT may be associated with lower risk of MI events, when compared to 5 national registries (Table 1). The observed MI incidence rate of 30 events per 100,000 persons was lower than any rate reported in the United States; the lowest rate was seen in the New York State Registry, which included 71.6 events per 100,000 persons. The Fukushima registry had an event rate of 37.9 per 100,000, despite stressors from the nuclear leak, though Japanese men are known to have lower rates of MI [10].

Unfortunately, the patients that did not qualify for testosterone therapy in the Low T Centers were not followed up and, therefore, could not be examined as controls. This study also relied on patient and family reporting of MI/stroke; this could have resulted in under- or overreporting, thereby possibly limiting the interpretation of these findings. Adverse outcomes were verified by interview and retrieval of medical records. The registries used as comparison cohorts do have limitations as they are not ideally matched controls. Admittedly, the Low T Center population is younger and has a lower prevalence of risk factors associated with cardiovascular events, such as hypertension, diabetes, and hyperlipidemia. To our knowledge, there are no similar low-risk and age-matched patient registries. It may be possible that patients seeking testosterone therapy are healthier, exercise more, and eat better than the general population. However, what is undeniable is that, even for a large practice of this size,

Table 3: Characteristics of study population from Low Testosterone (T) Centers.

	N	Percent
Patients on testosterone therapy	19,968	100%
Gender		
All male	19,968	100%
Age		
>55 years	3,833	19.2%
45–54 years	7,008	35.1%
35–44 years	6,829	34.2%
<34 years	2,296	11.5%
Prevalence of DM	798	4%
Prevalence of HTN	2,995	15%
Prevalence of HLD	2,396	12%
Mean age (years)	46.10	N/A
Drug		
Testosterone cypionate	18,742	93.8%
AndroGel	540	2.7%
Testim	326	1.6%
Fortesta	47	0.2%
Axiron	230	1%
Striant	9	0.04%
Others	74	0.66%
Hematocrit >52%	13,178	66%
Hematocrit ≤52%	8,785	44%
	Mean	Range
Time of exposure to testosterone (months)	24.7	0–60 month
Total testosterone level on treatment (ng/dL)	543 (SEM = 1.53)	50–1600
Calculated free testosterone on treatment (ng/mL)	13.08 (SEM = 0.48)	3.29–28.2
Estradiol level while on treatment (pg/dL)	31.1	6.4–453
PSA while on treatment (ng/mL)	1.22	0.02–109

treating only hypogonadism, very few cases of MI and stroke were reported.

Both Vigen et al. and this study performed retrospective chart review. However, only Veterans in the VA system were included in Vigen et al.'s article. These were not just Veterans but very ill patients with suspicion of heart disease, requiring the need for cardiac catheterization to elicit whether they had blocked coronary arteries. In the Low T Centers, most patients were healthy with few comorbid conditions, as implied by the low prevalence of risk factors for heart disease for hypertension (15%), diabetes (4%), and hyperlipidemia (12%) relative to the Veteran population [22]. In addition, the current study population was approximately 15 times the size of the Vigen et al. study, lending greater power to examine the study questions. There is a well-known limitation of studies using ICD-9 codes. The paper by Vigen et al. used ICD-9, but their data may not have been accurately classified. Oddly, some women were included in this study. In this present study, to avoid such problems, three nationwide telephone conferences groups were held with all providers, educating them on the correct codes required, to review all patients and update the ICD-9 as appropriate. Curiously, the mean level of total testosterone in Vigen et al.'s study was 332 ng/dL, and, by some definitions, this level reflects undertreatment or even a hypogonadal state. In contrast, the present study observed a mean of 543 ng/dL for treated patients. National guidelines for treatment of hypogonadism were followed. It could be argued that the patients with lower mean total testosterone levels achieved in the Vigen et al. study were themselves a risk for MI and stroke. Mean age of patients in the Vigen et al. paper was 63 years. In contrast, only 3 percent of the Low T Center patients were above 65 years.

Limitations to the Finkle et al. study include short duration, limited to 90 days, making it difficult to draw firm conclusions over time. In the Low T Centers, the follow up was up to 5 years. The Finkle et al. study was based on insurance data and included a heterogeneous group of patients using different treatment protocols. In contrast, treatment protocols at the Low T Centers are standardized throughout the different US centers. Testosterone levels were not reported in this study, which made us query whether these patients were actually treated or even reached eugonadal levels. The Low T Centers treat only men removing potential gender misclassification.

Baillargeon et al. [23] studied testosterone therapy in older men and examined the risk of MI in a population-based cohort of older men receiving intramuscular testosterone. They used a 5% national sample of Medicare beneficiaries. 6355 patients treated with at least 1 injection of testosterone were matched to a cohort of testosterone nonusers at a 1 : 3 ratio. They found that receiving testosterone therapy was not associated with an increased risk of MI (hazard ratio [HR] = 0.84; 95% C.I. = 0.69–1.02). But, for men in the highest quartile of the MI prognostic score, testosterone therapy was associated with a reduced risk of MI (HR = 0.69; 95% C.I. = 0.53–0.92). The similarity of these findings with the present study was that injectable testosterone was used. However, this study had younger patients with fewer comorbidities. In addition, this study followed a similar protocol of treatment. It may be argued that testosterone can protect from MI and strokes, if these criteria of injectable therapy and a fixed protocol were followed for a younger population.

Corona et al. [24] performed a meta-analysis of placebo controlled randomized controlled trials on the effect of testosterone on sexual function. No difference in the incidence of cardiovascular diseases was reported. When testosterone was compared to placebo for a comparison of cardiovascular diseases, the HR was 0.606 (95% C.I. = 0.157–2.335).

A review by Carson and Rosano [25] looked at available clinical trial data, indicating that the use of testosterone in middle-aged to elderly men does not increase cardiovascular risk nor does it unfavorably modify cardiovascular risk profile. They concluded that prospective data from large, well-designed, long-term trials of testosterone treatment are lacking and will be required to verify the cardiovascular efficacy/safety of chronic treatment.

Kelly and Jones [26] concluded that testosterone replacement in men diagnosed with hypogonadism, for whom mid-normal range levels are achieved, has a beneficial effect

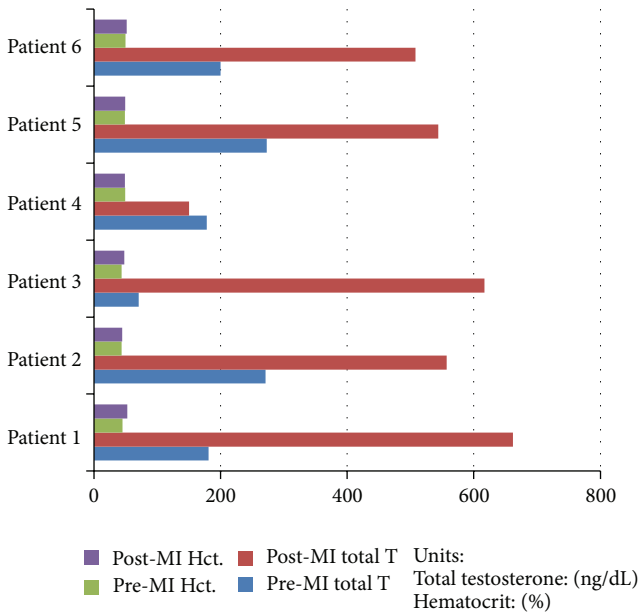


Figure 4: Changes of testosterone levels and hematocrit in patients with MI during treatment.

on several cardiovascular risk factors including cardiac ischemia, functional exercise capacity, and improved mortality. Yet studies in which patients were either undertreated or given high-dose testosterone have been associated with an increased risk of cardiovascular-related events. Clinical monitoring and titration of testosterone dose is therefore of paramount importance.

In a prospective study with testosterone undecanoate (TU), TU reduced fasting glucose, waist circumference, and improved surrogate markers of atherosclerosis in hypogonadal men with MS. Resumption and maintenance of T levels in the normal range of young adults determine a remarkable reduction in cardiovascular risk factors clustered in MS without significant hematological and prostate adverse events [27].

Our study reveals that MI does not appear to have a relationship with testosterone levels. Although the mean testosterone of patients with MI was lower by 62.5 ng/dL, we cannot infer that lower total mean testosterone levels led to the MI. The patients had established risk factors for MI. However, it may be fair to say that MI was not associated with higher levels of testosterone. Of the 6 cases with MI, the mean hematocrit was 48.97 and the mean initial hematocrit was 46.65 with a mean change of 2.32. There does not appear to be an association of MI to rise in hematocrit as the rise in patients without MI was similar or slightly higher, with mean change of 2.5 (Figure 4).

5. Conclusions

It appears from our study that practitioners should not only carefully screen and treat patients with hypogonadism but also carefully and closely monitor them. If patients are given T medications, compliance must be ensured. Only approximately 19,968 of our patients screened met criteria for treatment and were then followed on a protocol requiring regular, repeat clinic visits on a weekly or fortnightly basis for the prime modality of treatment with short acting injections. This strategy may have accounted for the positive outcomes that our study found. In addition, guidelines on treatment of hypogonadism (Endocrine Society, AACE, ISSAM, etc.) should be closely followed. It is important to ensure that patients reach therapeutic levels and monitor not only PSA and prostate but also the hematocrit. Our observation that the mean level of total testosterone was significantly higher in our study than the earlier study could be supportive of the association with low numbers of MI and stroke in testosterone-treated patients. Other comorbid risk factors such as diabetes, hyperlipidemia, and hypertension have also to be controlled, which appears to be the case with the close follow-ups, given our low rates of these comorbidities. Patients should be also given lifestyle change education. The flood of media advertisement may have pushed many patients into taking testosterone, but practitioners should not be reticent to deny treatment if patients do not qualify or have contraindications. It is important to spend time educating patients and telling them about the benefits and risks [28]. As no drug is completely safe for everyone, it is up to the practitioner to decide based on the understanding of risks/benefits by the patient and with informed consent.

Although the results of this study are promising, more research is recommended to further determine whether testosterone is cardioprotective or if it can cause MI or stroke in certain patient populations. A large randomized, controlled study powered in tens of thousands will be needed. To compare, the Women's Health Initiative had 161,800 patients, studied in a prospective manner [29].

These data suggest that treatment of low testosterone patients, particularly in the context of younger healthy men, does not lead to an increase of MI or stroke. This study population was considerably healthier even at baseline and may not be representative of the average hypogonadal patient. Furthermore, this study cannot claim that testosterone is protective against MI or stroke events given the lack of comparability of registries examined. In the Low T Center settings examined, use of testosterone was deemed safe, particularly for men under 65 years old without preexisting risk factors for heart attack or stroke. Low testosterone can be a public health issue, which, if left untreated, can lead to increased morbidity [30, 31].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Polycystic Ovary Syndrome: Important Underrecognised Cardiometabolic Risk Factor in Reproductive-Age Women

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder amongst women of reproductive age. Although PCOS is diagnosed exclusively based on reproductive criteria, it is also a metabolic disorder. Insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, obesity, and dyslipidemia are more common in women with PCOS than in age-comparable women without PCOS. Many of the metabolic abnormalities that manifest in PCOS are worsened by the concurrent incidence of obesity. However, some of these metabolic perturbations occur even in lean women with PCOS and therefore are rightfully recognized as intrinsic to PCOS. The intrinsic factors that produce these metabolic disturbances are reviewed in this paper. The consequences of obesity and the other metabolic aberrations are also discussed. The metabolic perturbations in PCOS patients lead to chronic low-grade inflammation and to cardiovascular impairments that heighten the risk of having cardiovascular disease. Even though many studies have shown an elevation in surrogate biomarkers of cardiovascular disease in PCOS women, it is still not clear to what extent and magnitude the elevation precipitates more frequent and earlier events.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder amongst women of reproductive age. It is a heterogeneous disorder of uncertain etiology, but there is strong evidence that complex interactions between genetic, environmental, and behavioral factors contribute to causing this syndrome [1]. PCOS affects as many as 10% of reproductive-age women when using the NIH criteria for diagnosis, and up to 18% of reproductive-age women are diagnosed with PCOS as per the Rotterdam criteria [2]. Nevertheless, at least 70% of PCOS cases remain undiagnosed in primary care [3].

Although the diagnosis of PCOS is based exclusively on reproductive criteria (hyperandrogenism, oligo/anovulation, and/or PCO on ultrasound) [4], and management tends to focus primarily on treatment of infertility and hirsutism [5], PCOS is also a metabolic disorder. Women with PCOS have an increased risk of presenting with insulin resistance (IR) [6], impaired glucose tolerance (IGT) [7], type 2 diabetes mellitus (DM2) [7], obesity [6], and dyslipidemia [8]. In addition to presenting with these traditional risk factors for CVD, women with PCOS also show evidence of an increase of nontraditional, novel CVD risk factors, such as subclinical atherosclerosis [9] and an elevation in inflammatory markers [10]. As PCOS seems to be dominated by metabolic consequences, both as a consequence of the condition and as a vector for further complications, including DM2, CVD, and the exacerbation of the reproductive features of the syndrome (hirsutism and an/oligoovulation) [5], it is evident that research on the metabolic and cardiometabolic features of PCOS is needed. The present review is a contribution to this overall effort. The sections that follow discuss the cardiometabolic aspects of PCOS, their potential causes, their associated risks, and possible screening measures.

Table 1: PCOS diagnostic criteria, adapted from Teede et al. 2010 [5].

NIH 1990	Rotterdam 2003	AE-PCOS Society 2006
Both of the following*: (i) chronic anovulation, documented by oligo-or amenorrhea (ii) clinical and/or biochemical signs of hyperandrogenism (with exclusion of other etiologies, e.g., congenital adrenal hyperplasia) with or without PCO on ultrasound	At least two of the following*: (i) chronic anovulation, documented by oligo-or amenorrhea (ii) clinical and/or biochemical signs of hyperandrogenism (iii) polycystic ovaries (by ultrasound)	(i) Clinical and/or biochemical signs of hyperandrogenism and at least one of the following*: (i) ovarian dysfunction (oligo/anovulation and/or polycystic ovarian morphology)

*After exclusion of the diseases that produce a similar clinical picture.

2. Methods

An extensive literature search was conducted to review publications from the late 1980s to the present. Online sources of medical databases included the US National Library of Medicine (NLM), the National Center for Biotechnology Information (NCBI) at the NLM, the Helios Group Central Medical Library, PubMed, and Medscape. The search was conducted with a combination of terms that included "PCOS," "cardiometabolic," "cardiovascular disease," "metabolic syndrome," "insulin resistance," and "obesity" that were assumed to be relevant. Articles were also selected among references in the published papers found in the automated searches. Studies and review articles covering the focused areas were then selected.

3. Diagnostic Criteria of PCOS and Different PCOS Phenotypes

Commonly Used Criteria. Three different sets of criteria have been used for the diagnosis of PCOS for the past two decades: the National Institutes of Child Health and Human Development (NICHD) or what is known as the NIH criteria (developed in 1990), the Rotterdam criteria (adopted at a PCOS consensus meeting held in 2003), and the Androgen Excess (AE) and PCOS Society (AE-PCOS) criteria (proposed in 2006) [5]. These criteria are summarized in Table 1.

The different diagnostic criteria create several phenotypes of PCOS. Even before the Rotterdam criteria were adopted, it was evident that different subgroups of PCOS existed and it was even suggested that these subgroups differed metabolically [6]. One extensive review by Moran and Teede sought to compare the metabolic profiles among these different reproductive phenotypes [11]. For simplification, the phenotypes were divided into four diagnostic groups: phenotype A (NIH PCOS of biochemical/clinical hyperandrogenism and oligo/anovulation with PCO); phenotype B (NIH PCOS of biochemical/clinical hyperandrogenism and oligo/anovulation without PCO); phenotype C (non-NIH PCOS with biochemical/clinical hyperandrogenism and PCO but with normal ovulation); phenotype D (non-NIH PCOS with oligo/anovulation and PCO but without any biochemical/clinical hyperandrogenism) [11]. The diagnostic criteria of each of these four phenotypes are summarized in Table 2.

Table 2: Diagnostic phenotypes of PCOS, adapted from Moran and Teede (2009) [11].

Phenotype A	NIH PCOS: hyperandrogenism and oligo/anovulation with PCO
Phenotype B	NIH PCOS: hyperandrogenism and oligo/anovulation without PCO
Phenotype C	Non-NIH PCOS: hyperandrogenism with PCO but with normal ovulation
Phenotype D	Non-NIH PCOS: no hyperandrogenism but with oligo/anovulation and with PCO

PCOS is also acknowledged as a metabolic disorder. Cardiometabolic features of PCOS are summarized as follows:

visceral obesity,

insulin resistance and hyperinsulinemia,

risk of type II diabetes,

disturbed secretion from adipocytes (adipokines, proinflammatory, and macrophage-derived factors),

dyslipidemia,

vascular endothelium dysfunction,

prothrombotic state,

atherosclerosis.

Most studies comparing the two subtypes of NIH PCOS, phenotypes A and B, report that women diagnosed under phenotype A present with few, if any, differences in metabolic profiles compared with women having phenotype B PCOS [11]. Similarly, most studies limiting comparison to only non-NIH subtypes, phenotypes C and D, also agree that women with phenotype C PCOS do not present with different metabolic risks compared to women with phenotype D PCOS [11]. However, most studies conclude that women with NIH PCOS (phenotypes A and B) present with more adverse metabolic profiles (including higher IR, increased prevalence of metabolic syndrome, and more adverse lipid profiles) than those with non-NIH PCOS (phenotypes C and D) [11].

Studies comparing women categorized under the NIH and non-NIH PCOS groups, but only after matching the subjects for BMI and WHR, found that the metabolic profiles (degree of IR, metabolic syndrome prevalence, and lipid profiles) are similar in the NIH and non-NIH PCOS women

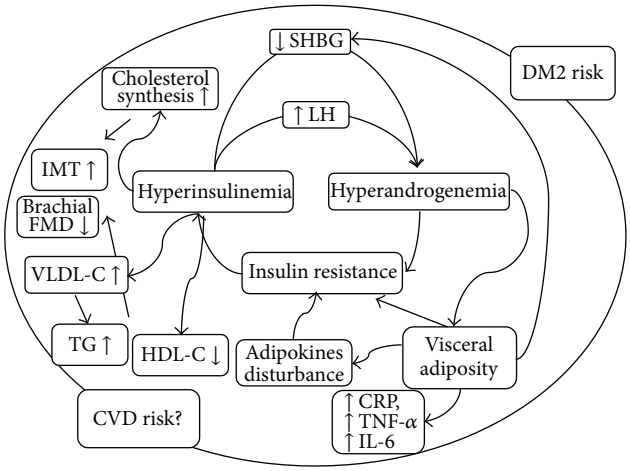

Figure 1: Pathophysiology of metabolic disturbances in PCOS. CVD, cardiovascular disease; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol; CRP, C-reactive protein; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; FMD, flow-mediated dilatation; IMT, intima-media thickness; SHBG, sex hormone binding globulin; LH, luteinizing hormone; DM, diabetes mellitus.

[11]. These results suggest that although NIH phenotypes present with more adverse metabolic profiles, the worse metabolic profile is not an inherent feature of NIH PCOS but is related to excess adiposity, particularly abdominal adiposity, which is more common in NIH PCOS groups [11]. The interactions between the different pathophysiologic factors of PCOS with metabolic syndrome are summarized in Figure 1.

The etiology of PCOS remains uncertain; however, due to a variety of predisposing genes that interact with environmental and lifestyle factors, PCOS is considered a complex genetic disorder [12]. A large number of population studies have focused on discovering genes that influence the development of PCOS using the candidate gene approach [13], but their findings have been mostly irreproducible. It is possible that a particular gene influences PCOS in one ethnic group but not in the others [12]. Various PCOS phenotypes presumably result from the interaction between multiple predisposing genomic variants, each exerting only minor effects, and strong environmental influences.

4. Cardiometabolic Aspects of PCOS

4.1. Obesity in PCOS. The prevalence of obesity among women with PCOS in the USA is 70 to 80%, almost twice as much as in the general US female population [6, 14]. Most studies report the prevalence of obesity in affected women outside the USA to be between 38 and 50% [6, 14]. Differences in diagnostic criteria, environmental factors, ethnicity, and lifestyle contribute to these variations [6, 15, 16]. Although less obesity is reported outside the USA, the prevalence of obesity among PCOS women outside the USA is still higher than that of women in the general population outside the USA [14]. As an example, 38% of Italian PCOS women are reported to be obese [14], but the reported prevalence of obesity in the general Italian female population is only 8% [17], highlighting a possible contribution of PCOS *per se*, in addition to lifestyle and other factors cited above, to the pathogenesis of obesity.

4.1.1. Consequences of Obesity in PCOS. Obesity plays a role in the expression of metabolic features and other clinical manifestations of PCOS [6, 8, 18]. IR appears in normal weight PCOS women, but the frequency and magnitude increase with obesity [19, 20]. The magnitude of IR, quantified in one study by the insulin to glucose ratio, demonstrated a strong, positive, and linear correlation with body mass index (BMI) of the PCOS subjects [20]. Hepatic insulin resistance, characterized by reduced sensitivity to insulin's suppression of endogenous glucose production, only occurs in obese PCOS women [21]. Obese PCOS women also have a 10-fold increase in their risk of suffering from DM2 and a 7-fold increase of IGT compared with normal weight (BMI $< 25\,\mathrm{kg/m^2}$) PCOS women [22]. An accelerated rate of conversion from IGT to DM2 is strongly dependent upon BMI [23].

Obesity in PCOS increases the patient's risk of developing cardiovascular disease. Among PCOS women, the prevalence of the metabolic syndrome, as in the general population, increases with increasing BMI and is highest in obese women with PCOS [17]. Studies report the prevalence of the metabolic syndrome in PCOS women in the USA to be 43–47%, twice more than in the age- and BMI-matched control population, suggesting that PCOS *per se*, possibly by promoting abdominal fat accumulation, increases the risk of acquiring the metabolic syndrome [17]. The effect of obesity, of causing chronic low-grade inflammation [24] with an elevation of inflammatory markers (such as CRP, TNF-α, and IL-6) that increase the risk of cardiovascular disease [25], is even more pronounced in PCOS. In PCOS and non-PCOS women, levels of TNF-α [26, 27], IL-6, and CRP [27] correlate directly with BMI, but overweight and obese PCOS women in some studies have presented with significantly higher levels of these inflammatory markers than their BMI-matched non-PCOS counterparts [10, 27].

4.1.2. The Pathogenesis of Obesity in PCOS. The pathogenesis of obesity in PCOS is likely multifactorial [28].

One study reported that, in a group of lean PCOS women, serum glycerol levels, which reflect lipolytic activity, were lower than glycerol levels in BMI-matched control women [28]. The same authors also showed that the subcutaneous adipocytes of the lean PCOS women were larger in size and exhibited a lower response to catecholamine-stimulated lipolysis than the adipocytes of the BMI-comparable control women, suggesting that decreased lipolysis of subcutaneous adipocytes is an early alteration in PCOS, leading to enlarged subcutaneous fat cells and later to the development of obesity [28].

Another contributor to the high prevalence of obesity in PCOS might be mutations in the peroxisome proliferator-activated receptor-γ gene [29]. A higher frequency of C $\rightarrow$ T substitution in exon 6 of the peroxisome proliferator-activated receptor-γ gene has been reported in PCOS

women than in BMI-matched controls [29]. This substitution enhances adipogenesis and increases the size of subcutaneous adipocytes, possibly leading to obesity [29].

Several studies also report that women with PCOS [30, 31] or with PCO morphology on ultrasound [32] have a higher prevalence of bulimic behavior, in part because of increased androgens [33], which increase appetite and inhibit impulse control [33]. After treatment with flutamide [34] or with antiandrogenic oral contraceptives [35], a reduction of binge eating and meal-related hunger, respectively, has been reported in bulimic women, supporting the idea that androgens may play a role in appetite dysregulation and in the development of obesity in PCOS [33]. Women with PCOS, in comparison to BMI-matched controls, also have reduced secretion of the gastrointestinal satiety peptide cholecystokinin [30] and have dysregulated secretion of the appetite-regulating gut hormone ghrelin [36, 37] that is independent of diet. These alterations may cause the reduction in satiety that has been reported by PCOS patients in comparison to BMI-matched control women [30, 37].

Additionally, it is widely believed that ghrelin's actions are mediated centrally by neuropeptide Y (NPY) and by the system of NPY fibers [36]. NPY acts centrally to increase appetite [38]. In a study on PCOS women, NPY levels were reported to be higher in obese and lean women with PCOS than in BMI-comparable control women [39].

4.1.3. The Distribution of Adipose Tissue in PCOS. Subcutaneous abdominal fat and visceral fat both contribute to the development of IR [40]. Visceral fat furthermore creates a chronic low-grade inflammation [41] and is a surrogate marker for ectopic fat accumulations [42], which are responsible for many of the harmful effects of obesity [43, 44]. Many studies, based on anthropometric measures such as the waist-to-hip (WHR) ratio, suggest that there is a tendency in PCOS towards the accumulation of fat in these harmful areas such as the abdominal visceral region. It is known that fat distribution in the abdominal area is associated with more adverse metabolic profiles in PCOS [11, 45]. However, there is debate as to whether abdominal fat storage occurs more in PCOS than in weight-matched controls. Several studies quantifying abdominal subcutaneous and visceral adipose tissue by MRI [45, 46] and DEXA (which only quantifies total central abdominal fat) [47] found no difference in the volume of total abdominal fat or visceral fat between PCOS women and BMI-matched controls. On the contrary, Dolfing et al. demonstrated even less visceral fat accumulation in lean PCOS women compared to matched controls assessed by MRI [48]. However, in other studies, MRI [42] showed an increase in visceral and subcutaneous abdominal fat, and DEXA [49] showed an increase in the proportion of upper body fat in PCOS women compared to BMI-matched controls. The different results may depend on the small number of patients and controls and may also be related to the degree of obesity [42]. Studies in which the majority [41, 42, 50] or all [49] of the women were nonobese have reported a higher quantity of central abdominal fat in PCOS women than in BMI-matched controls. Results from a more highly powered study with over 200 patients and controls evaluated by DEXA support the suggestion that the disparities reported by different authors are related to the degree of obesity [41]. When the 220 subjects from the study were stratified according to BMI into obese and nonobese subgroups, there was no difference in the quantity of central abdominal fat between obese PCOS women and obese control women, but when limiting the comparison to the nonobese women, the quantity of central abdominal fat was higher in nonobese PCOS women compared to the quantity in nonobese control women [41]. These observations demonstrate that when obesity is present, most subjects display abdominal obesity, independently of being afflicted with PCOS or not [41]. However, when obesity is not present, PCOS patients stock a higher portion of their total adiposity in the abdominal region than do BMI-comparable controls [41]. Abdominal adiposity may therefore be a risk factor in nonobese PCOS women that confers on them adverse metabolic profiles compared to their BMI-matched non-PCOS counterparts [41]. Studies report that the quantity of central abdominal fat positively correlates with the degree of IR in nonobese PCOS women [41] and to the level of inflammatory markers [50]. DEXA accurately quantifies fat in different regions, is not operator dependent, and, unlike MRI, can be used on large populations [41]. DEXA may therefore be a useful screening tool for nonobese PCOS women susceptible to central abdominal fat accumulation and, hence, to the adverse metabolic complications associated with centripetal fat distribution [11].

4.2. Insulin Resistance and Hyperinsulinemia. Insulin resistance (IR) occurs in 30% of lean women with PCOS [8] and 95% of obese women with PCOS [51]. The presence of IR in Mediterranean populations of PCOS patients is somewhat less than that reported in other nations [52, 53]. South Asians in particular have high prevalence of insulin resistance and metabolic syndrome with central obesity in comparison with other PCOS-related ethnic groups of a similar BMI [54]. African American and Hispanic women are more obese and more prone to metabolic problems [55, 56]. The ethnic origin and cultural habits largely contribute to manifestations and risks of insulin resistance in PCOS [7]. Overall, 60–80% of women with PCOS present with elevated insulin levels [57–60].

Insulin is also a major regulator of many enzymes involved in lipoprotein metabolism [61, 62]. Resistance to insulin may contribute, in part, to the dyslipidemia observed in PCOS [16, 61]. A detailed description of the steps involved in lipoprotein metabolism is beyond the scope of this review. However, it has been proven that IR increases the hepatic secretion of VLDL and decreases the elimination of VLDL and of chylomicrons [19]. The persistence of VLDL and of chylomicrons in the circulation provides a major source for triglyceride (TG) production [62]. IR also leads to the more rapid clearance of apolipoprotein-a, a constituent of HDL-C, thus reducing the production and levels of HDL-C [62]. In several population-based studies, described in a paper by Miccoli et al., measures of IR correlated positively with levels of TG and VLDL-C and negatively with levels of HDL-C [62]. A study by Slowinska-Srzednicka and colleagues sought to

elucidate the role of insulin resistance in the development of lipid abnormalities in women susceptible to PCOS [63]. In a group of women with polycystic ovaries, after adjustment for age, BMI, and sex hormones, regression analysis showed a strong positive association between fasting insulin levels and TG and VLDL-C levels and a negative association between fasting insulin levels and levels of the HDL constituent apolipoprotein-a [63].

The high prevalence of insulin resistance in PCOS also renders PCOS women 10 times more likely than controls to develop gestational diabetes and up to 5 times more likely to develop insulin-related complications such as spontaneous abortion [8].

4.2.1. The Pathogenesis of Insulin Resistance in PCOS. The IR of PCOS is in part independent of obesity; it is primarily a result of intrinsic factors. A postbinding decrease in the phosphorylation of the tyrosine residues and an increase in the phosphorylation of the serine residues of the intracellular domain of the insulin receptor cause resistance to insulin's metabolic actions [6]. An elevation in serine phosphorylation not only decreases the responsiveness of the insulin receptor to its substrate, but also enhances the activity of P450C17, the key enzyme of adrenal and ovarian steroid synthesis [64]. The same defect in serine phosphorylation is therefore thought to cause both IR and hyperandrogenism in a subgroup of PCOS patients [6]. Other possible causes of insulin resistance in PCOS include increased serine phosphorylation of the adaptor protein IRS-1 [6]. Serine phosphorylation of the latter disrupts intracellular signaling necessary for the translocation of GLUT4 into the plasma membrane [6]. Reduced expression of GLUT4 has been demonstrated in the plasma membranes of adipocytes of both lean and obese PCOS patients [65]. Increased activation of ERK1/2 pathways in muscle cells of PCOS women may also be responsible for resistance to insulin's metabolic actions [6, 66]. Although ERK1/2 pathways are usually involved in insulin's mitogenic actions [6, 66], enhanced basal activation of ERK1/2 can also inhibit the IRS-1 pathways necessary for GLUT4 translocation to the plasma membrane [66]. Increased lipolysis in visceral fat cells may contribute to the hepatic insulin resistance observed in obese PCOS women [67]. Visceral fat cells of PCOS women demonstrate an enhanced lipolytic response to catecholamines [67]. Enhanced lipolysis of visceral fat raises fatty acid and glycerol delivery to the portal vein and liver, perturbing liver function, eventually leading to hepatic IR, as well as to hepatic inflammation and to interference with the production of SHBG [67].

4.3. Impaired Glucose Tolerance and Type 2 Diabetes. The American Diabetes Association has designated PCOS as a nonmodifiable risk factor for type 2 diabetes [68]. The prevalence of IGT and DM2 in women with PCOS, assessed in three large ethnically diverse US cross-sectional studies, was 23–35% for IGT and 4–10% for DM2, that is, twice the prevalence in age- and weight-matched healthy women without PCOS [6]. The prevalence of IGT and DM2 among PCOS women from other countries (Italy, Netherlands) was also found to be significantly higher than the prevalence in control women from the same region, although the overall proportion of European PCOS women having IGT or DM2 is nevertheless lower than that of US PCOS women affected by these conditions [6]. Authors suggest that different diagnostic criteria, diet, race, and ethnicity may account for the higher prevalence of IGT and DM2 in US PCOS women [6, 16]. When the PCOS women from European and US studies were stratified according to BMI and comparisons were limited to women in comparable BMI categories, the differences in the prevalence of IGT and DM2 between US and European PCOS women still persisted but decreased, highlighting the contribution of lifestyle to the disparities in IGT and DM2 observed between US and European women [6]. Additionally, a study of two PCOS populations in the USA, one urban ethnically diverse and one rural ethnically homogeneous, showed similar proportions of women with IGT and DM2 in each of the two populations, therefore demonstrating that PCOS may be a more important risk factor for IGT and DM2 than factors such as race and ethnicity [69]. These general tendencies towards a deterioration of glucose metabolism have been confirmed by a meta-analysis indicating a higher prevalence of IGT (odds ratio 2.54) and DM2 (odds ratio 4) in PCOS women than in BMI-matched controls [7].

Studies have also reported higher conversion rates from normal glucose tolerance (NGT) to IGT and from IGT to DM2 in PCOS women [70, 71]. IGT is an independent predictor of developing DM2 and CVD and of suffering mortality from CVD [71]. Early identification and treatment of IGT with lifestyle intervention and/or metformin have been shown to improve outcomes [72]. These observations have led experts at the most recent ESHRE/ASRM-sponsored PCOS consensus workshop to suggest the implementation of an annual screening of all PCOS women for IGT with the OGTT [3, 73], the most sensitive test for assessing IGT in PCOS [6].

Chronic hyperinsulinemia *per se* exacerbates IR, leading to a higher demand for insulin production and eventually to β-cell burnout, thereby accelerating the progression to IGT and DM2 [5]. Although women with PCOS have higher basal insulin secretion conditioned by chronic IR, they demonstrate β-cell secretory defects, manifested by reduced insulin secretory response to meals [74] and eventually an overall secretion of insulin that is inadequate for the degree of IR [75]. IR, β-cell secretory defects, and eventual β-cell burnout contribute to the development of IGT and DM2 in PCOS [74].

4.4. Adipokines. The increased incidence and severity of cardiovascular risk factors and of metabolic disturbances in PCOS may be in part related to the abnormal production and release of adipokines and inflammatory factors by adipose tissue [8]. Although traditionally regarded as a storage organ, emerging evidence also strongly suggests that adipose tissue is an endocrine organ [8], whose altered function may produce widespread cardiometabolic disturbances in PCOS. It is believed that dysregulated adipocyte function and obesity play a pathophysiological role in PCOS [5, 28].

4.4.1. Leptin. Leptin, a protein secreted by adipocytes, suppresses an individual's appetite and promotes energy expenditure [76]. Serum leptin levels are elevated in obese patients, who are considered leptin resistant [76]. Hyperleptinemia seems to be a positive risk factor for cardiovascular disease [77–79]. Although some studies have found leptin levels to be elevated in PCOS women compared to controls [80, 81], the general consensus reported by the majority of published studies is that there is no difference in circulating leptin levels in PCOS subjects in comparison to BMI-matched controls [82–90]. The different results might be explained by differences in ethnicity, heterogeneity in criteria used to classify PCOS, and low number of PCOS subjects and controls [90, 91].

Most studies report that adiposity, quantified by BMI, is the main correlative component and determinant of leptin levels in PCOS women [82–90]. Leptin mRNA expression in adipocytes did not differ between PCOS women and BMI-matched controls [89], providing further evidence that obesity, rather than PCOS *per se*, affects leptin production and circulating levels. After adjustment for BMI, some authors report that leptin levels do correlate minimally with the free androgen index [82, 83, 86, 92] but nevertheless do not differ between visibly hirsute and nonhirsute women with PCOS [82]. Most studies addressing leptin and insulin report that, after adjustment for BMI, leptin levels in PCOS women do not correlate with the chronic insulin levels [82, 85, 88, 89, 92], while others report that leptin levels in PCOS women did correlate with measures of insulin resistance [81, 83]. However, in further support of findings that negate a significant correlation between insulin resistance and leptin levels, treatment of chronically hyperinsulinemic insulin resistant PCOS women with the thiazolidinediones troglitazone [85] or rosiglitazone [88] was shown to lower insulin levels but did not alter leptin levels in these patients.

4.4.2. Adiponectin. Adiponectin, which is secreted exclusively by adipose tissue, exerts insulin sensitizing actions both indirectly [93] and directly by activating tyrosine phosphorylation of the skeletal muscle insulin receptor [94]. Adiponectin levels are reduced in insulin resistance states such as DM2 across all ethnic groups [95]. Low levels are also associated with a faster progression towards DM2 in at-risk individuals [95] and with higher risk of CHD in women [96]. Low levels are also possibly associated with high LH/FSH ratios and impaired ovulation because adiponectin in normal levels reduces secretion of LH through AMPK phosphorylation without affecting FSH secretion [97].

A meta-analysis has demonstrated that adiponectin levels are lower in PCOS women than in control women of comparable BMI [98]. A more recent meta-analysis has indicated that the T45G polymorphism in the adiponectin gene is associated with PCOS [99]. Although few studies exist focusing on high molecular weight adiponectin and the earlier meta-analysis did not specifically evaluate levels of high molecular weight (HMW) adiponectin [98], which is considered to be a more potent mediator of insulin sensitivity [100], it has been reported that levels of HMW adiponectin and the ratio of HMW adiponectin to total adiponectin are both lower in PCOS women than in age- and BMI-comparable controls [101].

4.4.3. Visfatin. Visfatin is a cytokine secreted, among other cell types, by adipocytes [102]. It stimulates glucose uptake by cells, thus inducing insulin-mimetic effects [102]. A meta-analysis established that plasma visfatin levels are significantly increased in subjects with obesity, DM2, metabolic syndrome, and CVD [103]. Furthermore, in diabetics, serum visfatin levels increase with progressive β-cell deterioration [104]. Haider et al. demonstrated that insulin inhibited visfatin release from adipocytes in healthy subjects, suggesting that elevated visfatin levels may reflect insulin resistance [105].

Given visfatin's insulin-mimetic actions, some authors have suggested visfatin may be elevated to compensate for insulin resistance [106] and to prevent further resistance to insulin [107, 108]. However, elevated visfatin levels may produce harmful effects. Rising visfatin levels correlate with the degree of endothelial dysfunction, quantified by the decline in flow-mediated vasodilation and impaired renal clearance [109]. Visfatin activates nuclear transcription factor NF-κB in vascular endothelial cells [110] and in lipid-laden macrophages of atherosclerotic lesions [111], culminating in the activation of metalloproteinase-2 [110] and metalloproteinase-9 [111, 112], leading to vascular inflammation and plaque destabilization, respectively.

In patients undergoing carotid endarterectomy or percutaneous coronary interventions, visfatin expression is higher in the atherosclerotic lesions of symptomatic patients than in the lesions of asymptomatic patients, further emphasizing the role of this adipokine in plaque destabilization and acute cardiovascular events [112]. Likewise, elevated visfatin levels in PCOS may also signal heightened cardiovascular risk in certain women with this syndrome, particularly in those with insulin resistance.

Given its associations with insulin resistance and vascular inflammation, several studies have been undertaken to elucidate the role of visfatin in PCOS. Higher levels of serum visfatin and visfatin mRNA in adipocytes have been reported in PCOS women compared to BMI-matched controls [106, 107, 113–115]. Serum visfatin levels were found to correlate with BMI [106, 113], insulin resistance [106, 107, 116], free androgen index [107], and LH levels [115].

It has been observed that metformin treatment for 3 months lowered visfatin levels [114]. However, the investigations demonstrated many interstudy variations in parameters such as BMI, IR, FAI, and LH that significantly correlated with visfatin levels in some studies but not in others. These variations may be attributed to the small number of participants, less than 30 PCOS women in all but one [107] of these investigations, and to interracial variations in the phenotypic expression of PCOS [106].

More recent studies have reported no differences in visfatin levels between PCOS women and controls [117, 118], therefore necessitating further inquiries with more participants to clarify the role of this adipokine in PCOS.

4.4.4. Chemerin. Chemerin is a chemotactic protein secreted by adipocytes [119, 120] that is necessary for adipocyte differentiation [121]. It is able to attract macrophages, which express the chemerin receptor CMKLR1 (chemokine-like receptor 1) [122]. In view of its chemoattractant properties, this adipokine may be one factor underlying the link between obesity and chronic inflammation [122]. Chemerin also induces insulin resistance in peripheral tissues such as skeletal muscle by activation of ERK-1/2 and NF-κB pathways, culminating in inhibited cellular glucose uptake [120]. Insulin stimulates chemerin secretion, promoting a vicious circle increasing insulin resistance [106]. This protein is thought to possibly present a link between obesity and diabetes [120]. Serum chemerin levels have been found to correlate with BMI [119, 120], WHR [119, 120], triglycerides [119], elevated blood pressure [119], and adipocyte volume [120]. The latter has been found to be higher in PCOS patients, even in lean PCOS patients when compared to BMI-matched controls [28]. Chemerin levels in PCOS are consequently of interest, as this may be one of the factors underlying the insulin resistance so common in PCOS. Chemerin is also implicated in inflammation, which may be responsible for vascular damage leading to CVD. Chemerin levels have been reported to be higher in obese PCOS women than in BMI- and WHR-matched controls [106, 123] as well as in lean PCOS women compared to BMI-matched controls [123]. Treatment of the PCOS patients with metformin for 6 months has lowered chemerin levels and improved insulin resistance, without changing BMI [106].

4.5. Proinflammatory and Macrophage-Derived Factors. This section presents a survey of macrophages and proinflammatory factors such as tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and interleukin-6 (IL-6).

4.5.1. Macrophages. Adipose tissue inflammation mediated by activated tissue macrophages (ATMs) is a major pathway culminating in the development of obesity-related insulin resistance [124, 125]. CD11c is a marker specific to these ATMs that infiltrate adipose tissue in obese individuals and secrete cytokines such as TNF-α and IL-6, both of which are associated with insulin resistance. In contrast, the markers CD206, CD14, and CD163 are expressed by less inflammatory macrophages [125]. CD11c-expressing macrophages cluster around dead adipocytes, forming histologically defined crown-like structures (CLS) [125]. The density of CLS has been found to correlate with the degree of insulin resistance and obesity [125]. CD11c and CLS density is significantly higher in lean and obese PCOS women than in BMI-comparable non-PCOS controls [42]. The observation that CD11c macrophages and CLS occur more frequently in obese men than in obese women has led to the suggestion that the increase observed in lean and obese PCOS women is likely a result of hyperandrogenism [42]. CD11c adipose tissue infiltration and CLS may be an early change in lean hyperandrogenic PCOS women leading to the development of insulin resistance in this group [42] that is comparable to the insulin resistance in obese controls [19].

4.5.2. Proinflammatory Factors. TNF-α plays a role in the pathogenesis of insulin resistance [126]. It inhibits tyrosine phosphorylation of the insulin receptor and of IRS-1 in muscle and fat cells [126] and has also been shown to down-regulate the expression of the GLUT4 transporter necessary for cellular entry of glucose [127]. Serum levels of TNF-α are elevated in both obesity and DM2 [126]. Studies report that TNF-α levels in PCOS correlate with BMI [26, 27, 50, 128] and that circulating TNF-α levels are elevated in both nonobese [26, 129] and obese [27] PCOS women when compared with BMI-matched controls. However, in other studies, these differences in the levels of TNF-α between PCOS women and controls diminished after adjusting for BMI and abdominal adiposity [50, 128], thus questioning whether TNF-α elevations are related to PCOS or are a function of excess adiposity. A meta-analysis found no significant difference in TNF-α levels between PCOS subjects and BMI-matched controls [10]. However, the authors caution against overinterpretation of these results, as their study also revealed evidence of a publication bias favoring publication of studies that underestimate the differences in TNF-α levels between PCOS women and controls [10]. An important difference between PCOS and control subjects may lie in the TNF-α receptor [130]. Most of the metabolic effects of TNF-α are mediated through the TNF-α receptor 2 [130]. Although TNF-α receptor 2 levels were increased in obesity, no differences in levels of this receptor were observed between PCOS women and controls [130]. However, a methionine 196 arginine polymorphism in exon 6 of the gene encoding the TNF-α receptor 2 was reported to be significantly more frequent in women with PCOS than in controls, suggesting that TNF-α plays a role in the development of metabolic pathologies in PCOS and that this might be related to a structural change in the TNF-α receptor that confers a more responsive phenotype, rather than to circulating TNF-α levels or to the density of TNF-α receptors *per se* [130]. Authors agree that larger and more highly powered studies are needed to clarify the role of TNF-α in PCOS [10, 130].

CRP has been proven to be a strong independent predictor of cardiovascular events in healthy asymptomatic as well as symptomatic women in the general population [131, 132]. Obesity is associated with elevations in CRP [133]. A meta-analysis of 26 studies matching carefully for BMI revealed that CRP is elevated in PCOS independently of obesity [10]. This elevation of CRP in PCOS is more pronounced when obesity is present, further heightening the risk of cardiovascular events in this group of women [10]. However, the authors caution against overattributing increased cardiovascular risk to PCOS *per se* because, after adjusting for BMI, the elevation in CRP attributable to PCOS is relatively small [10].

Interleukin-6 (IL-6) is released by mononuclear leucocytes and adipose tissue [10], with levels being elevated in obesity [10]. It directly stimulates hepatic CRP synthesis [10]. Although IL-6 elevations have been reported in lean and obese PCOS women in relation to BMI-comparable controls [10], a recent meta-analysis proved no significant difference in circulating IL-6 levels between PCOS women and BMI-matched controls [10], suggesting that elevated IL-6 in PCOS is primarily related to obesity. However, a promoter region

polymorphism (G/C) at position -174 of the gene encoding IL-6 has been found to be strongly associated with DM2 in the Caucasian population [134]. This same polymorphism has been reported to occur more frequently in PCOS patients [135, 136]. Furthermore, a certain microsatellite CA-repeat polymorphism in the locus encoding the α-subunit of the IL-6 receptor is associated with obesity, while the Arg148 allele in the region encoding the gp130 subunit of the IL-6 receptor gene is more common in normoandrogenic subjects than hyperandrogenic ones [137]. These observations suggest that genetically determined hypersignaling defects in the IL-6 receptor, rather than only circulating IL-6 levels, may be implicated in the pathogenesis of metabolic hyperandrogenic disorders such as PCOS [137]. However, relatively little is known about this field and authors agree that larger studies are needed [10, 137].

4.6. Dyslipidemia. Dyslipidemia is the most common metabolic abnormality in PCOS [4, 8], and polycystic ovary syndrome is the leading cause of dyslipidemia in reproductive-age women [138]. Observations of PCOS affected women and their relatives have shown that the probability of developing dyslipidemia is 1.8-fold larger in the PCOS individuals [8]. Overall, studies of PCOS patients report slightly decreased levels of cardioprotective HDL-C, with slightly elevated levels of TG, VLDL-C, and LDL-C [4, 8]. PCOS women display the lipid profile observed in insulin resistant states such as DM2 and characterized specifically by elevated TG and lowered HDL-C [8, 71]. The main determinant of heart disease risk is the total cholesterol (TC) to HDL-C ratio [51]. This ratio is also slightly elevated in PCOS patients [8].

Elevated LDL-C [71, 139–141] and VLDL-C [139] in PCOS are further elevated when excess adiposity is present, but, as confirmed by a recent meta-analysis, the higher levels occur in PCOS independently of obesity [142]. Elevated LDL-C levels are linked with hyperandrogenemia [139, 140, 143]. It is unclear whether hyperandrogenemia and elevated LDL-C have a causal relationship or whether these are closely related genetic traits inherited together [140].

Although LDL-C and VLDL-C are elevated in PCOS independently of obesity, obesity is thought to be the major determining factor for elevations in TG levels and for the reduction of HDL-C levels that are observed in PCOS [71, 140, 142, 144]. In a study comparing lipid profiles between PCOS probands, their sisters with and without PCO morphology on ultrasound, and controls, the elevated TG levels and reduced HDL-C levels in probands relative to the other groups disappeared after controlling for BMI [144], suggesting that BMI is the predominant determinant of TG and HDL-C levels in PCOS [144], both of which are strong independent risk factors for death from cardiovascular disease [145]. However, after adjustment for BMI, age, and centripetal obesity in another large study of PCOS women, HDL-C levels still remained significantly lower in PCOS women when compared to controls, though only slightly so [146]. This indicates that factors other than BMI and centripetal obesity are contributors to the lowering of HDL-C levels in PCOS women, although, of course, BMI is observably a significant determinant of lipid profiles [146].

In a study that did not control for diet, HDL-C levels were unexpectedly higher in obese PCOS women than in obese controls [71]. This inconsistency signals that, in addition to BMI [144], other factors such as age, ethnicity, genetic influences, and environment also modulate lipid profiles of women with PCOS [15, 71, 141, 147]. The importance of environmental (diet and activity level) and genetic contributions to dyslipidemia is evidenced by the fact that TG elevations in American PCOS women compared to Italian PCOS women persist even after controlling for BMI [15, 147].

Even PCOS women with normal lipid profiles may be at increased risk of cardiovascular events. This is because significantly higher levels of lipoprotein-a and a higher proportion of small, dense LDL have been found in PCOS patients compared to controls [148], although TC and total LDL-C levels did not differ between PCOS and control women [148]. This similarity in TC and total LDL-C levels makes the PCOS women appear to have normal lipid profiles. Such PCOS women are at higher risk of cardiovascular events, because certain lipoproteins, such as lipoprotein-a and small, dense LDL-C, are more atherogenic [148].

Another atherogenic shift in PCOS is the lipid-to-protein ratio of HDL-C particles. The lipid-to-protein ratio in an HDL-C particle reflects the capacity of the particle to remove cholesterol from tissues [149]. A reduction of this ratio signals a decreased or impaired capacity to remove cholesterol and prevent atherosclerosis [149].

In one study, the lipid-to-protein ratio in the HDL-C was found to be lower in obese PCOS women than in obese women without PCOS [149]. The latter finding signals a drop in the antiatherogenic properties of HDL-C of PCOS women [149].

Despite slight changes in lipid profiles in PCOS, most women with PCOS are young and have normal blood pressure and hence do not qualify for primary prevention of cardiovascular disease [8]. Nevertheless, performing at least one measurement of lipid profiles in PCOS in conjunction with an assessment for other cardiovascular risk factors such as smoking and family history of CVD is suggested [8].

4.7. Traditional and Novel Cardiovascular Risk Factors in PCOS

4.7.1. Traditional Cardiovascular Disease Risks Factors in PCOS. Traditional risk factors for cardiovascular disease, such as IGT, DM2, dyslipidemia, obesity, and elevated blood pressure, are more prevalent in women with PCOS than in control women of similar age [51].

4.7.2. Markers of Atherosclerosis. Calcification of the coronary arteries assessed by electron beam computed tomography correlates with the degree of atherosclerosis found on histopathological exam and was found to predict the incidence of cardiovascular events in asymptomatic women [150]. The prevalence and extent of coronary artery calcification (CAC) were found by several studies to be higher in

both younger (aged 30 to 45 years) and older (aged over 40 years) women with PCOS than in controls, independently of age and BMI [151–153]. It has been suggested that the reported increase in CAC among PCOS women is related to the parameters that were different in PCOS women in relation to the control women in the studies: increased LDL-C [151, 153], lower HDL-C [153], and hyperinsulinemia [153]. Among the women with PCOS, BMI was a significant predictor of whether the women would have CAC [151–153], leading to the suggestion that obese women with PCOS should be targeted for aggressive treatment and prevention of cardiovascular disease [151, 152]. Talbott et al. also reported a higher prevalence and extent of aortic calcification (AC) in women with PCOS [153]. The investigators of the latter study found that total testosterone was an independent risk factor for greater AC [153]. In animal models, testosterone exacerbated atherosclerosis in female monkeys but conferred a protective effect in males [153]. Similarly, a large study reported that men with the highest total testosterone levels had a reduced risk of AC, but, conversely, women with elevated testosterone levels had the highest risk for CAC [153, 154], leading authors to suggest that the aorta in women may be more sensitive to the effects of endogenous testosterone [153, 154].

Increased intima-media wall thickness (IMT) is an early marker of atherosclerosis [155]. Increased carotid intima-media wall thickness (CIMT) is also a strong independent predictor of the occurrence of major cardiovascular events later in life [155]. Higher CIMT has been reported in both younger (age 20 to 35 years) [57, 156] and older (over 45 years) [153] patients with PCOS in comparison to controls of similar age and BMI. A recent meta-analysis indicated that women with PCOS had a 0.072 to 0.084 mm higher CIMT compared to controls [157]. Every 0.10 mm increase in CIMT has been estimated to increase the risk of a myocardial infarction (MI) by 15% and the risk of stroke by 18% [157]. The increase in CIMT in PCOS relative to controls of comparable age and BMI has been associated in different studies with higher levels of insulin [57], hyperandrogenism [156], Il-18 [157], LDL-C [157], and abdominal obesity [157], although the contribution of each of these factors to increased CIMT in PCOS has not been systematically evaluated [157]. However, CIMT increases with age in PCOS, as in the general population [157].

4.7.3. Vascular Endothelial Dysfunction. Several studies have demonstrated decreased brachial artery flow-mediated dilation (FMD), a marker of endothelial function, in young normal weight, overweight, and obese women with PCOS compared to body mass matched controls [9, 17]. The decreased FMD was observed even in normal weight PCOS women who were also normotensive and had normal lipid profiles [9] and who therefore lacked many of the traditional cardiovascular risk factors [9]. It is considered that elevated androgen levels in the PCOS women relative to controls contribute to the observed decline in endothelial function [57].

An earlier study by Paradisi et al. further supports the role of elevated androgen levels in precipitating endothelial dysfunction [158]. When obese PCOS and control women of similar age, BMI, LDL-C, and TC levels received intrafemoral artery infusions of the endothelial-dependent vasodilator methacholine chloride (MCh), the leg blood flow was 50% less in the PCOS women compared to the controls, suggesting impaired nitric oxide (NO) production in the endothelial cells of PCOS women [158]. The degree of decrease in leg blood flow was strongly associated with free testosterone levels [158]. This, in conjunction with the observation that androgen deprivation in men has enhanced endothelial-dependent vasodilation [159], initially led to the suggestion that elevated androgen levels in PCOS women may be a major contributor to endothelial dysfunction and macrovascular disease [158].

Several molecules implicated in endothelial dysfunction have been linked to PCOS. A recent meta-analysis indicated that homocysteine, a mediator of endothelial injury, is in higher levels in PCOS women than in controls of similar age and BMI [160]. The same study also demonstrated that levels of asymmetric dimethylarginine (ADMA), a competitive inhibitor of endothelial NO synthase and an independent risk marker for cardiovascular morbidity and mortality [161], are higher in PCOS women than in age- and BMI-matched controls [160]. Several studies have found that, in comparison to age- and BMI-comparable control women, PCOS women also exhibit elevated levels of endothelin-1 [6, 57], a by-product of endothelial damage and a potent vasoconstrictor [57]. Plasminogen activator inhibitor-1, which inhibits fibrinolysis and in higher levels predisposes to accelerated development of atherosclerosis [162], has been shown to be elevated in normal weight young PCOS women relative to controls [163].

4.7.4. Coagulation and Fibrinolytic Disturbances. Disturbances in circulating markers of coagulation and fibrinolysis may contribute to cardiovascular disease risk. Several studies observed dysregulation of the hemostatic system, particularly hypofibrinolysis, hypercoagulability [163, 164], and endothelial and platelet dysfunction, in women with PCOS [164, 165]. The potential mechanisms of coagulation disturbances remain to be elucidated. It has been observed that women with PCOS have high circulating concentrations of PAI-1 and fibrinogen, independent of age and BMI, correlated with low SHBG and high insulin levels [166]. Hyperinsulinemia impairs fibrinolysis by enhancing PAI-1 secretion and by inhibiting hepatic production of SHBG [167]. Our group recently observed strong negative linear association between serum SHBG and CRP levels, even following adjustment for BMI, WHR, TT, HOMA-IR, total cholesterol, LDL cholesterol, and triglyceride levels [168]. High CRP and sE-selectin were recently observed as the strongest explanatory factors of high fibrinogen levels in women with PCOS [166]. Strong positive correlation is recognized between hyperandrogenism and hypofibrinolysis in women with PCOS contributing to a prothrombotic state [163].

4.7.5. Cardiac Dysfunction. Studies report that, compared to age- and BMI-matched controls, young PCOS women

have increased left ventricular mass index (LVMi) [61], a predictor of CVD morbidity and mortality [8], and decreased diastolic filling [61, 169]. Both of these abnormalities occur independently of excess weight, presenting in lean as well as overweight and obese PCOS patients. Additionally, decreased left ventricular ejection fraction has been reported in young overweight and obese women with PCOS compared to controls [61].

4.7.6. The Risk of Cardiovascular Events in PCOS. Even though many studies have shown an elevation in surrogate biomarkers of cardiovascular disease in PCOS women, the question remains as to what extent this translates into more frequent or earlier events. Only a few prospective epidemiological studies have addressed this question.

A 49-year follow-up study of 786 women diagnosed with PCOS based only on ovarian wedge section found that the risk of fatal cardiovascular events was not different between PCOS women and controls [170]. Similarly, a prospective 21-year follow-up study of 31 women with histologically verified Stein-Leventhal syndrome found that although the CVD risk factors hypertension and hypertriglyceridemia were still more prevalent among the PCOS women in postmenopausal age, these women did not have an increased risk of suffering MI, stroke, or death caused by CVD compared to non-PCOS women [171]. A retrospective study of 319 women diagnosed with PCOS based on more stringent criteria (an/oligoovulation and hyperandrogenism) reported that there was no difference in cardiovascular mortality risks between PCOS women and age-matched controls [172], although the PCOS women demonstrated a higher risk of nonfatal cerebrovascular events, even after adjustment for BMI [172].

Another investigation followed 82,439 women aged 20–35 for 14 years [173]. Compared with women reporting a history of regular menses, women reporting a history of very irregular menses had a significantly higher risk of nonfatal and fatal cardiovascular disease, even after adjustment for BMI, age, menopausal status, and smoking [173]. Although these women were not diagnosed with PCOS, it is estimated that 80–90% of women reporting menstrual irregularity have PCOS [8]. Furthermore, a recent meta-analysis indicated a 2-fold increased risk of coronary heart disease (CHD) and stroke for patients with PCOS relative to women without PCOS [174]. The meta-analysis found that there is a 55% increase in the risk for CHD and stroke in PCOS women using only studies that adjusted for BMI, showing that BMI is not the sole cause of increased risk of cardiovascular events in women with PCOS [174].

The risk of cardiovascular events also appears to be higher in postmenopausal women with a history of PCOS than those without. The Women's Ischemia Syndrome Evaluation study reported that the cumulative 5-year event-free survival for women without a history of PCOS is 88.4% and only 78.9% for those with a premenopausal history of PCOS [175].

A retrospective study found similar results. The study evaluated the incidence of CV events (MI, angina, heart failure, stroke, and CV death) in a cohort of 2300 PCOS women between 1988 and 2009 [176]. Overall, CV events were not any more prevalent in the cohort than in the local female population [176]. However, when the cohort was stratified by age and comparisons were limited to age-similar groups in the local female population, PCOS showed an association with CV events within each age group [176]. The age-specific prevalence of CV events was significantly higher in PCOS patients over 45 compared with the local female population, with odds ratio as high as 12.88 in women over 65 with a premenopausal history of PCOS [176]. Factors in the cohort associated with an increased risk of CV events were age, hypertension, obesity, smoking, and having DM2 [176].

5. Conclusions

This review has provided a survey of many intrinsic causative factors that underlie IR, obesity, and the other metabolic perturbations associated with PCOS. Future investigations may elucidate which of these intrinsic causative factors are present in the different phenotypic subgroups of PCOS women. Further studies may also delineate which of these intrinsic factors are more common in certain geographic regions and associated ethnicities. Finally, and more importantly, through future investigations we may gain an understanding of which of these causative factors are associated with the most severe consequences. This may help foster a better understanding of the pathophysiology underlying PCOS in different subgroups and populations. Such knowledge could then be leveraged to devise the most optimal screening and effective management for women from different subgroups and ethnicities.

Experts have advocated the annual screening of all PCOS women for IGT with the OGTT and more frequent screenings for those with other DM2 risk factors, such as a family history of DM2. Some investigators have also suggested screening lean women with DEXA for excess abdominal fat accumulation, as lean PCOS women with a higher proportion of abdominal fat relative to BMI-comparable control women are more susceptible to developing insulin resistance and may therefore benefit from more aggressive prevention.

Central abdominal fat accumulation has been associated with insulin resistance, chronic inflammation, and harmful ectopic fat accumulations. Studies evaluating abdominal fat accumulation in PCOS women relative to BMI-comparable controls have reported contradictory results. This highlights the need for more studies that would quantify ectopic, visceral, and subcutaneous abdominal fat by CT or MRI, in order to provide definitive answers about the relationship between PCOS and visceral fat.

Uncertainty also remains in PCOS regarding the incidence of cardiovascular disease later in life, despite the indisputable presence of multiple CV risk factors earlier in the lifespan. Therefore, prospective observational trials are urgently needed that follow patients diagnosed with PCOS based on strict inclusion criteria and that track these women from a young age until after menopause.

The most urgent problem with the current management of PCOS is that many doctors focus on the short-term cosmetic and reproductive consequences, while metabolic and psychological risks are often not considered. Indeed, current

knowledge clearly indicates that metabolic complications are present in both lean and obese women with PCOS. Early screening and close follow-up are therefore encouraged in both groups of patients.

Also, the observed trends of insulin resistance incidence and early cardiovascular event occurrences within families point to the conclusion that the family members of PCOS women should probably be screened for metabolic disturbances.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Epicardial Adipose Tissue Is Nonlinearly Related to Anthropometric Measures and Subcutaneous Adipose Tissue

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Introduction. Adipose tissue is the largest endocrine organ, composed of subcutaneous (SAT) and visceral adipose tissue (VAT), the latter being highly associated with coronary artery disease (CAD). Expansion of epicardial adipose tissue (EAT) is linked to CAD. One way of assessing the CAD risk is with low-cost anthropometric measures, although they are inaccurate and cannot discriminate between VAT and SAT. The aim of this study is to evaluate (1) the relationship between EAT thickness, SAT thickness and anthropometric measures in a cohort of patients assessed at the cardiology unit and (2) determine predictive power of anthropometric measures and EAT and SAT thickness in establishment of CAD. *Methods*. Anthropometric measures were obtained from 53 CAD and 42 non-CAD patients. Vascular and structural statuses were obtained with coronarography and echocardiography, as well as measurements of the EAT and SAT thickness. *Results*. Anthropometric measures showed moderate positive correlation with EAT and SAT thickness. Anthropometric measures and SAT follow nonlinear *S curve* relationship with EAT. Strong nonlinear *power curve* relationship was observed between EAT and SAT thinner than 10 mm. Anthropometric measures and EAT and SAT were poor predictors of CAD. *Conclusion*. Anthropometric measures and SAT have nonlinear relationship with EAT. EAT thickness and anthropometric measures have similar CAD predictive value.

1. Introduction

The most common chronic disorder nowadays is obesity, which is defined as excessive accumulation of adipose tissue (fat), traditionally defined with body mass index (BMI) exceeding 30 kg/m^2. The majority of adipose tissues in the human body are deposited as subcutaneous or visceral fat, differing in their structure and function [1]. Subcutaneous adipose tissue (SAT) is primarily located on the extremities, while visceral adipose tissue (VAT) is located around internal organs. Adipose tissue has important endocrine role, as genetic analyses have shown that adipose tissue (especially VAT) expresses numerous secretory proteins (adipocytokines) [2]. Recently it was established that proinflammatory adipocytokines secreted by thickened epicardial adipose tissue (EAT), VAT located around the heart and coronary arteries, lead to the development of CAD [3, 4]. Obesity is recognized as risk factor for coronary artery disease (CAD) [5, 6]. Particularly risk-increasing is abdominal obesity, characterized with predominantly abdominal accumulation of fat (VAT) [7, 8]. Abdominal obesity is key characteristic for establishment of the metabolic syndrome, together with any two of the following: hypertriglyceridemia, reduced high density lipoprotein (HDL), raised blood pressure, and disorders of carbohydrate metabolism (raised fasting plasma glucose or diabetes type 2) [9]. Since BMI is general indicator of obesity, measures of abdominal obesity, such as waist

circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHR), are suggested as more accurate in describing the distribution of body fat compared. Ultrasound anthropometric indicators, such as SAT and EAT thickness, have been recently proposed as methods of assessment of body fat distribution. Research on relationships between "classic" and ultrasound anthropometric indicators is limited [10].

Coronary heart disease is the leading cause of death worldwide. Coronary angiography is the criterion standard for detecting significant flow-limiting stenosis and direct imaging of atherosclerotic changes in coronary arteries. Because of the inherent limitations, disadvantages and complications of coronary angiography, attention has been directed toward using physiologic, noninvasive modalities to determine the severity of coronary stenosis. Epicardial adipose tissue thickness has been reported as a marker for the presence and severity of coronary artery disease [3, 4, 11], superior to WC and WHR.

Due to limited research in the field, we performed the detailed analysis of the relationship between anthropometric measures (WC, hip circumference (HC), WHR, BMI, and waist-to-height ratio) and echocardiographically obtained EAT and SAT thickness in a cohort of patients admitted at the cardiology unit. We also tested power of these "classical" and ultrasound anthropometric measures for the prediction of CAD.

2. Methods

2.1. Study Population. In this study 95 Caucasian subjects were included, 55 males and 40 females, all of whom signed an informed consent. Subject's age spanned from 31 to 80 years, with BMI of 19.9 to 38.7 kg/m^2. Due to clinical symptoms all subjects underwent echocardiography and coronarography at the Cardiology Department of Clinical Hospital Center Osijek, Croatia. Exclusion criteria were diabetes and chronic renal failure.

2.2. Epicardial and Subcutaneous Adipose Tissue Thickness Measurement. Ultrasound measurements of EAT thickness were performed with Siemens Acuson V70 and linear probe L10 (5–11 MHz) and SAT thickness with probe P4-2 (all Siemens Medical Solutions, Malvern, PA, USA). EAT thickness was assessed using M mode in long parasternal axis with subject in left lateral decubitus position. The EAT was identified as echo-free space between the myocardial wall and the visceral layer of pericardium in end-diastole above right ventricle [3, 4, 11, 12]. Subcutaneous adipose tissue thickness was measured above umbilicus while the subject was on his/her back. All measurements were done in triplicate with probe repositioning by one sonographer.

2.3. Anthropometry. Anthropometric measures were taken in all subjects in standing position, while being barefoot on a flat surface, after exhaling, at a level parallel to the floor (waist circumference, hip circumference, height), using a stretch-resistant measuring tape to the nearest 1 mm. All anthropometric measures were measured to one decimal. Waist circumference was measured at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest. Hip circumference was measured around the widest portion of the buttocks. Body weight was measured using electronic calibrated scales to the nearest 100 grams, while subjects wore minimal clothing. Each measurement was repeated twice.

Table 1: Anthropometric measures and EAT and SAT thickness in CAD and non-CAD subjects.

	CAD	Non-CAD
Number	53	42
Age (years)	62 ± 10	62 ± 13
BMI (kg/m^2)	27.90 ± 3.08	27.03 ± 5.30
Waist circumference (cm)	98.84 ± 12.19	89.95 ± 12.82*
Hip circumference (cm)	98.96 ± 9.39	93.32 ± 12.59*
Waist-to-hip ratio	0.99 ± 0.08	0.96 ± 0.05*
Waist-to-height ratio	0.57 ± 0.06	0.53 ± 0.08*
EAT thickness (mm)	5.25 ± 2.50	3.89 ± 1.76*
SAT thickness (mm)	15.38 ± 6.67	12.32 ± 8.33*

CAD: coronary artery disease; BMI: body mass index; EAT: epicardial adipose tissue; SAT: subcutaneous adipose tissue. *$p < 0.05$: difference between the groups.

2.4. Statistical Analysis. Data analysis was performed using SAS software (version 8.02, Cary, NC, USA). Data in tables were reported with mean value and a standard deviation. Normality of distribution was tested using the Shapiro-Wilks test. Group comparisons were done using Mann-Whitney test and Student's t-test (CI of 95%). Correlation was used to explore the nature of relationships among variables. Curve estimation models were used to assess the relationships among anthropometric variables, EAT and SAT thickness. ROC analysis was used to determine predictive capabilities of anthropometric variables EAT and SAT thickness for CAD. Accepted statistical significance was for $p < 0.05$.

3. Results

Subjects were assigned to CAD group ($n = 53$) if they had ≥50% narrowing of one or more coronary arteries or to control, non-CAD group ($n = 42$), in which different valvular abnormalities were established. Subjects in the groups were of similar age and BMI. Larger values of waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), waist-to-height ratio (WHtR), EAT thickness, and SAT thickness were observed in CAD patients (Table 1).

Epicardial adipose tissue thickness showed weak to moderate correlation with WC and HC and weak correlation with BMI, WHtR, and SAT, while SAT showed stronger correlation with BMI, WC, HC, and WHtR. Neither EAT nor SAT correlated with age and WHR.

Table 2: Receiver Operating Characteristic (ROC) analysis for prediction of coronary artery disease (CAD) with anthropometric measures and ultrasonographically obtained epicaradial and subcutaneous adipose tissue thickness.

Tested variable	AUC	p	95% CI
BMI	0.605	0.082	0.481–0.729
Waist circumference	0.688	0.002	0.579–0.797
Hip circumference	0.643	0.018	0.528–0.758
Waist-to-hip ratio	0.657	0.009	0.545–0.770
Waist-to-height ratio	0.673	0.004	0.559–0.786
EAT thickness	0.658	0.009	0.548–0.768
SAT thickness	0.634	0.027	0.511–0.756
Overall model	0.751	<0.001	0.651–0.834

Overall logistic regression model for CAD classification with anthropometric measures and EAT and SAT as predictor variables was tested with ROC analysis. AUC: area under curve; CI: confidence interval; BMI: body mass index; EAT: epicardial adipose tissue; SAT: subcutaneous adipose tissue. Coronary artery disease (CAD) was defined as ≥50% narrowing of one or more arteries.

3.1. Nonlinear Relationships between the Variables. Relationship between anthropometric variables (except WHR) and EAT thickness was described the best as a nonlinear *S curve* relationship (Figures 1(a)–1(e)). An *S curve* model was also the best fit for EAT and SAT thicknesses' relationship (Figure 1(f)). Visual inspection of scatter plots revealed that SAT thinner than 10 mm had a different relationship with EAT than SAT thicker than 10 mm. Relationship between SAT thinner than 10 mm and EAT thickness was described the best as *power curve* relationship, since the *power curve* model explained 65.1% of variance ($p < 0.001$) (Figure 2). When SAT thickness was above 10 mm, no relationship with EAT thickness was established, as all regression models were nonsignificant (Figure 2).

3.2. Receiver Operating Characteristic Analysis. Receiver operating characteristic (ROC) curves for CAD classification with anthropometric variables and EAT, SAT thickness showed that all predictor variables, except BMI ($p = 0.082$), can detect CAD ($p < 0.05$), but with poor accuracy and similar sensitivity and specificity. Anthropometric variables and EAT and SAT had similar areas under curve. Using logistic regression for CAD prediction, overall model with anthropometric measures, SAT and EAT thickness as predictor variables were created and tested using ROC analysis. The overall model had moderate accuracy and the largest area under curve (AUC 0.751), while areas under curve for assessed measures ranged from 0.605 to 0.688 (Table 2).

4. Discussion

In our study cohort, all anthropometric variables, except WHR, showed moderate positive correlations with ultrasound measures of EAT and SAT. Such finding is obvious, since energy intake and expenditure disproportion lead to accumulation of body fat at all locations but also means that used anthropometric variables cannot discriminate between EAT and SAT. Lack of correlation and relationship of EAT and SAT with WHR might indicate that WHR is not valuable anthropometric index for prediction of EAT and SAT. It was also reported previously that several other anthropometric measures are superior for assessment of body fat distribution than WHR [13–15].

All anthropometric variables (except WHR) and SAT thickness, independently of each other, showed a nonlinear *S curve* relationship with EAT thickness in all analyzed subjects (Figure 1). As classic anthropometric measures measure both VAT and SAT, observed nonlinear relationship of EAT and classic anthropometric measures might be a "blurred picture" of an *S curve* clearly evident in relationship between EAT and SAT. Interestingly, in patients with SAT thinner than 10 mm, we observed strong nonlinear *power curve* relationship with EAT (explaining 65.1% of the variance), while SAT thicker than 10 mm is independent of EAT thickness, since we could not establish any linear or nonlinear relationship (Figure 2). This unexpected findings, along with the average maximum thickness of EAT (5.25 ± 2.50 mm), indicate that EAT does not accumulate above certain thickness regardless of increase in total adipose tissue volume.

Adipocytes in different compartments of adipose tissue enlarge by storing excess lipids, but when certain threshold overall volume of adipose tissue is reached, they cannot accumulate lipids anymore. It was shown that every person enters early adulthood with specific number of adipocytes, which can only be hypertrophy when accumulation of lipids occurs, while hyperplasia of precursors is strictly regulated [16]. Therefore, it is reasonable to predict that certain volume of adipose tissue has corresponding threshold values of different anthropometric measures.

Adipose tissue expands and undergoes extensive remodeling during positive caloric balance [16]. Adipose tissue remodeling occurs under inflammation and is characterized with relatively inadequate angiogenic remodeling and accumulation of extracellular matrix and immune cells [16]. It was reported that hypertrophy of the adipose tissue is associated with inability of recruitment and/or differentiation of existing preadipocytes into mature adipocytes, leading to overfill of adipocytes with lipids (lipid spillover) [17, 18]. Lipid spillover, through release of free fatty acids, acts as an inductor of endoplasmic stress and toll-like receptor type 4 pathways, responsible for the inflammation of the adipose tissue [19]. The enlargement of adipocytes leads to macrophage infiltration [17, 18, 20]. Curat et al. [21] showed, *in vitro*, that leptin (mostly produced by SAT) was responsible for macrophage extravasation, important step in initiation of adipose tissue inflammation [22]. Along with lipid spillover and leptin secretion, adipocyte hypertrophy is associated with hypoxia, a known inductor of inflammation [23]. Hypoxia acts through hypoxia induced factor 1, transcription factor that leads to extensive extracellular remodeling of adipose tissue, thus hampering angiogenesis, which is a rate limiting step in adipose tissue expansion [24]. Hypoxia occurs as adipocyte grows above 150–200 μm in diameter, thus exciding oxygen diffusion distance [25]. These pathophysiological processes could explain observed nonlinear relationship of EAT and other measures, indicating that EAT undergoes

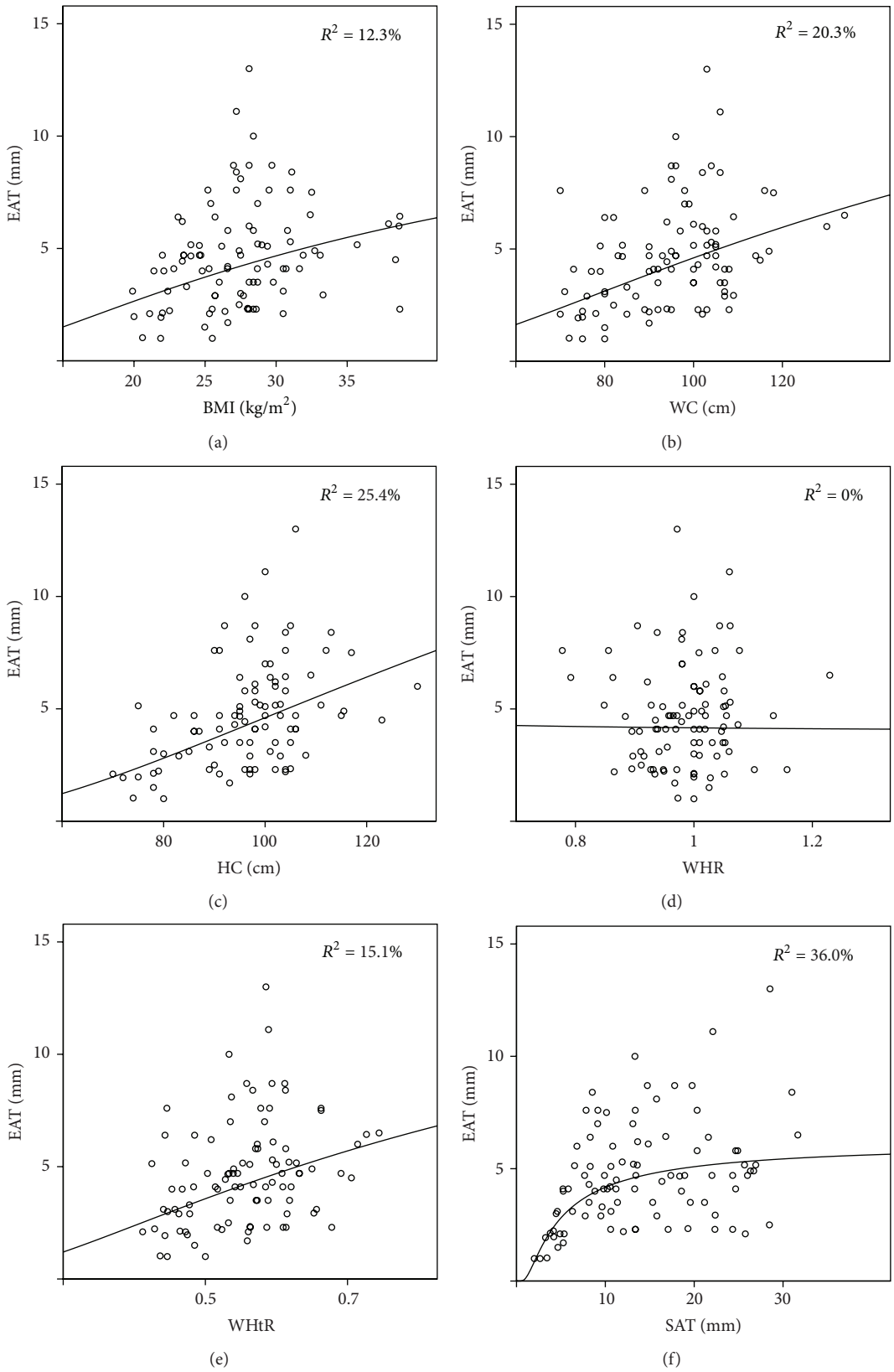

Figure 1: Nonlinear *S curve* relationship of EAT thickness with anthropometric measures and SAT thickness.

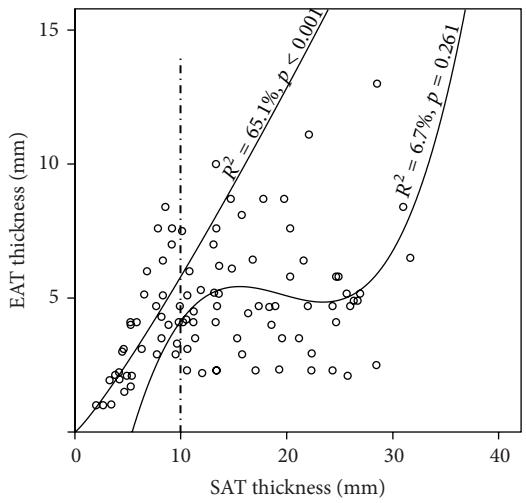

Figure 2: Nonlinear *power curve* relationship of EAT thickness and SAT thinner than 10 mm.

remodeling and inflammation, thus hindering its ability to expand.

Moreover, accumulation of total adipose tissue leads to general low-grade inflammation, resulting in development of atherosclerosis [26]. Inflammation and atherosclerosis are the result of interplay of different cytokines, with important role of adipocytokines secreted by adipose tissue [26]. Coronarography is considered as standard for diagnosing CAD. Since it has several disadvantages and complications, complementary methods for assessing status of the coronary arteries would be useful. Anthropometric measures and SAT and EAT had poor accuracy at discriminating between CAD and controls in our study sample. The combined, overall model of anthropometric measures and SAT and EAT thickness had moderate accuracy at discriminating CAD and non-CAD patients. Since increased EAT thickness has been linked to CAD [3, 4, 11, 12], EAT thickness was expected to classify CAD patients better. Poor predictive accuracy of EAT thickness for CAD might be explained with current study setting, where control group consisted of patients with valvular diseases as controls. Recently, atherosclerotic pathophysiological process that results in CAD was associated with the aortic stenosis, the most common valvular disease [27]. Visceral obesity and its proinflammatory adipocytokines and subsequent inflammation have been linked with aortic stenosis, so called valvulometabolic risk [28]. Decreased levels of anti-inflammatory adiponectin have been linked with greater valvular inflammatory activity [29], while increased levels of leptin have been found in blood of the valvular patients [30]. Systemic effects of different compartments of adipose tissue could contribute to development of aortic stenosis and other valvular disease, as well as potential paracrine effects of the EAT and periaortic adipose tissue. Therefore, similar pathophysiological background of CAD and valvular disease might explain poor predictive accuracy of EAT thickness in our study sample, where the proportion of our control patients had aortic stenosis. Nevertheless, further research on role of EAT is warranted, since, in healthy individuals, it protects the myocardium while its expansion might lead to lipotoxicity and generation of proinflammatory cytokines [31]. Imaging MRI and echocardiographic studies showed that myocardial fat and cardiac dysfunction (i.e., left ventricle LV overload, systolic dysfunction, and hypertrophy) are associated with accumulation of EAT [32, 33]. Our study is limited to lack of expression profile of inflammation markers in adipose tissue and blood, as well as a relatively small sample size.

In conclusion, in cardiac patients, the EAT thickness follows a nonlinear relationship with anthropometric measures and SAT thickness. Ultrasound measures of EAT thickness have similar predictive accuracy for CAD to anthropometric measures of visceral obesity.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Coronary Microvascular Function and Beyond: The Crosstalk between Hormones, Cytokines, and Neurotransmitters

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Beyond its hemodynamic function, the heart also acts as a neuroendocrine and immunoregulatory organ. A dynamic communication between the heart and other organs takes place constantly to maintain cardiovascular homeostasis. The current understanding highlights the importance of the endocrine, immune, and nervous factors to fine-tune the crosstalk of the cardiovascular system with the entire body. Once disrupted, this complex interorgan communication may promote the onset and the progression of cardiovascular diseases. Thus, expanding our knowledge on how these factors influence the cardiovascular system can lead to novel therapeutic strategies to improve patient care. In the present paper, we review novel concepts on the role of endocrine, immune, and nervous factors in the modulation of microvascular coronary function.

1. Coronary Microcirculation and Coronary Flow Reserve: Definition and Clinical Relevance

Obstructive disease of the epicardial coronary arteries was recognized as the cause of angina pectoris more than 2 centuries ago, and sudden thrombotic occlusion of an epicardial coronary artery has been established as the cause of acute myocardial infarction since over 100 years [1]. Cardiovascular diseases such as stable and unstable angina, acute myocardial infarction, peripheral artery disease, and stroke may be related to the loss of the protective properties of the endothelium, which in normal conditions preserves vascular tone, and inhibit thrombosis and inflammation [2]. Endothelial dysfunction, an early and reversible event in the pathogenesis of atherosclerosis, is associated with increased vascular smooth muscle tone, arterial stiffening, and intima-media thickness. As reported by Hirata et al., recent studies have shown that the severity of endothelial dysfunction correlates to the risk of primary or recurrent cardiovascular events. Moreover, a growing number of interventions known to reduce cardiovascular risk, such as physical exercise or alimentation, also improve endothelial function [3, 4]. Thus, the authors conclude that it is possible to consider the endothelial function as a "barometer" of cardiovascular health useful to direct patient management and evaluation of therapeutic strategies. The coronary microvasculature (vessels <300 μm in diameter) cannot be directly imaged *in vivo*, but a number of invasive and noninvasive techniques can be used to assess parameters that depend directly on coronary microvascular function [5]. Among other vascular beds, the endothelial function can be assessed also at the level of the coronary circulation by mean of the coronary flow reserve (CFR) [6]. From a pathophysiological point of view, reduced CFR can result from the combination of different alterations such as impaired vasodilation, enhanced vasoconstrictor responsiveness, and/or structural remodeling of the coronary microvasculature. Thus, the functional status of the coronary microcirculation can be assessed by testing endothelium-dependent and endothelium-independent vascular responses [7]. Adenosine, dipyridamole, and papaverine are often used to trigger arteriolar vasodilation and hence increase coronary

blood flow, mainly by a direct relaxing effect on vascular smooth muscle cells (endothelium-independent effect) [8]. On the other hand, acetylcholine (Ach) administration causes either vasodilation in the presence of a healthy endothelium able to stimulate nitric oxide (NO) production or vasoconstriction via the stimulation of muscarinic receptors on vascular smooth muscle cells [9] in the absence of a functional endothelium. Like Ach, also bradykinin and substance-P are mediators commonly used to test endothelial-dependent vasorelaxation [10].

It is important to notice that a high hemoglobin value, a major determinant of whole blood viscosity, predicts cardiovascular events [11]. Erythrocyte deformability is a key rheologic feature to allow blood flow, especially in the capillaries. Sandhagen and Lind [12] evaluated the relationships between blood viscosity, erythrocyte deformability, coronary risk, and endothelial vasodilatory function: rheological factors (such as heightened plasma viscosity and increased red blood cell aggregation) modify blood fluidity. A reduced fluidity may limit the microcirculatory flow due to the viscus resistance [13]. Figure 1 summarizes the key factors which contribute to the coronary microvascular function.

Coronary anatomy and myocardial blood flow are major determinants of clinical symptoms and survival in patients with epicardial coronary artery and coronary microvascular diseases (as in case of X syndrome or "stress cardiomyopathy") [14–17]. The CFR, defined as maximal hyperemic flow divided by resting flow, is measured by echocardiography and by other techniques (coronary angiograms and fractional flow reserve, positron emission tomography, and magnetic resonance imaging), each one with distinct advantages and limitations [2]. An important distinction needs to be made between techniques that directly measure coronary blood flow (e.g., positron emission tomography) and those measuring blood flow velocity (e.g., Doppler catheters), from which coronary velocity reserve is only calculated [18].

CFR represents the ability of the coronary flow to increase above its basal value when the coronary vascular bed is maximally dilated. It is a global parameter of coronary flow, which is early altered in the presence of a coronary microvascular dysfunction/disease or epicardial coronary artery stenosis. It is possible to study coronary flow in all main coronaries by transthoracic Doppler echocardiography; however, normally the left anterior descending artery is the coronary of choice. CFR is defined as the ratio of maximal hyperemic to basal diastolic coronary velocity. Maximal hyperemic flow is obtained during adenosine infusion. Under normal physiological conditions, α-adrenergic vasoconstriction in the heart is suppressed by myogenic, endothelial, or metabolic factors, with the most important being NO [19]. Buus et al. demonstrated in healthy subjects that adenosine-induced myocardial hyperemia is partly dependent on an intact endogenous NO production suggesting that adenosine-mediated vasodilation is partly endothelium dependent [20]. Thus, as we and others confirmed [21–25], a decrease in myocardial perfusion reserve may be caused by endothelial dysfunction.

There are different potential applications of this technique since CFR allows for the assessment of the hemodynamic relevance of a moderate and severe coronary stenosis, the detection of coronary restenosis, or upstream coronary occlusion. Moreover, evaluation of the CFR is of crucial importance in order to appreciate myocardial reperfusion or "no-reflow" following reopening of the infarct-related artery [2]. This technique gives also the possibility of noninvasive follow-up of arterial bypasses [2].

CFR measurement by transthoracic Doppler echocardiography reflects coronary microvascular function, as a cost-effective and noninvasive screening test in many conditions (age and sex, hypertension, diabetes, hypercholesterolemia, cardiomyopathies, valvular heart diseases, heart transplantation, endocrine, and immunitary diseases). Thus CFR represents a simple but at the same time very important tool to investigate the physiology and pathophysiology of heart and systemic diseases. Furthermore, it is also helpful in evaluating therapeutic interventions and prognosis-risk stratification in cardiomyopathies [26], coronary artery disease, and heart transplantation [2, 27–29].

Coronary microvascular dysfunction, defined as reduced coronary flow reserve and/or coronary endothelial dysfunction, is associated with a 2.5% annual major adverse event rate that includes death, nonfatal myocardial infarction, nonfatal stroke, and congestive heart failure [30]. Early identification of microvascular coronary disease by echo-CFR or other coronary reactivity tests may be beneficial in prognosis evaluation and patient stratification for optimal medical therapy [31]. This is of paramount importance because many diseases, that is, endocrine, metabolic, and immune conditions, affect vascular and in particular coronary function.

2. Hormonal Influences on Vascular Reactivity: At the Heart of Coronary Microvascular Dysfunction

Endothelial cells integrate several different stimuli (Figure 2) to maintain the appropriate coronary tone and flow able to always match the variable myocardial demands. Here we describe the role of hormones and growth factors in this complex crosstalk.

2.1. Vitamin D. Vitamin D deficiency has been associated with prevalence and incidence of cardiovascular (CV) disease, suggesting a role for bioregulators of bone and mineral metabolism in CV health. In the absence of major cardiovascular risk factors, Vitamin D deficiency is a frequent finding in essential hypertension patients and is independently associated with left ventricular hypertrophy [32]. In this setting, endothelial Vitamin D receptor plays an important role in endothelial cell function and blood pressure control, regulating angiotensin II effects with its action being related to endothelial NO synthase expression [33].

Vitamin D deficiency leads to secondary hyperparathyroidism, a condition associated with CV disease [34]. Parathyroid hormone (PTH) is an important regulator of calcium homeostasis, and its elevation increases prevalence and incidence of CV risk factors and disease, including CV mortality and vascular structural abnormalities [34].

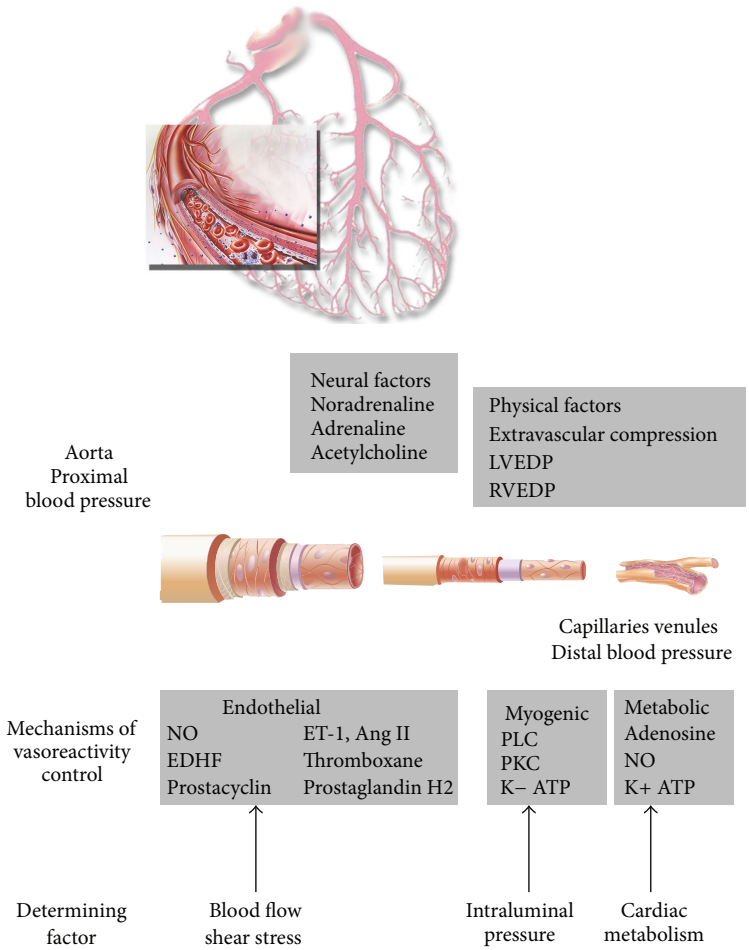

Figure 1: Physical, metabolic, and neural factors modulate microvascular coronary blood flow [9]. The coronary blood flow (CBF) is driven by the pressure difference between the aortic sinus and the coronary sinus (or the right atrium pressure). In the absence of obstructive stenoses, the epicardial arteries offer very little (10%) resistance to CBF and serve mainly as conductance vessels. Capillaries and venules are likewise responsible for only 10% of CBF resistance and mainly function as capacitance vessels, holding 90% of the total myocardial blood volume. Under normal conditions and to a large extent also under pathological conditions, coronary vascular resistance is primarily controlled by the prearterioles (vessels of 500 mm in diameter) and arterioles (200 mm). The prearterioles are epicardial (extramyocardial) vessels that react to changes in shear stress and intravascular pressure to preserve adequate perfusion pressure in the distal arteriolar bed. They are responsible for 25% of the total coronary vascular resistance. The arterioles are the true intramyocardial regulatory component of the coronary circulation and these vessels represent the largest proportion (55%) of the total coronary vascular resistance. Endothelium-dependent vasoreactivity prevails in the larger arterioles (100–200 mm in diameter) and translates flow-related stimuli into vasomotor responses, that is, vasodilation with increase in flow and vice versa. Medium-sized microvessels (40–100 mm in diameter) react predominantly to intraluminal pressure changes sensed by stretch receptors located in vascular smooth muscle cells (myogenic control, through signals mediated by phospholipase C and protein kinase C and calcium homeostasis); that is, they constrict when the intraluminal pressure increases and, conversely, dilate when the pressure decreases. Finally, the tone of the smaller arterioles (vessels of 40 mm in diameter) is modulated by the metabolic activity of the myocardium. As such, increased metabolic activity leads to vasodilatation of the smaller arterioles, which leads to pressure reduction in the medium-sized microvessels and myogenic dilation, which, in turn, increases flow upstream resulting in endothelium-dependent vasodilation. These mechanisms effectively and efficiently allow the microcirculation to regulate myocardial perfusion both at rest and at different levels of myocardial metabolic demand. LVEDP: left ventricular end diastolic pressure; RVED: right ventricular end diastolic pressure; ET-1: endothelin-1; ANG II: angiotensin II; NO: nitric oxide; EDHF: endothelial derived factor; PLC: phospholipase C; PKC: protein kinase C (modified from [9]).

The higher risk persists after adjusting for Vitamin D levels, renal function, and standard risk factors. Thus, PTH represents an important CV risk factor with an independent predictive value for CV disease and mortality [35]. Furthermore, accumulating evidence suggests bidirectional interplay between PTH and aldosterone (which is a well-known independent CV risk factor [36]). This interaction may lead to a disproportionally increased risk of CV damage and metabolic and bone diseases. PTH stimulates aldosterone secretion by increasing the calcium concentration in

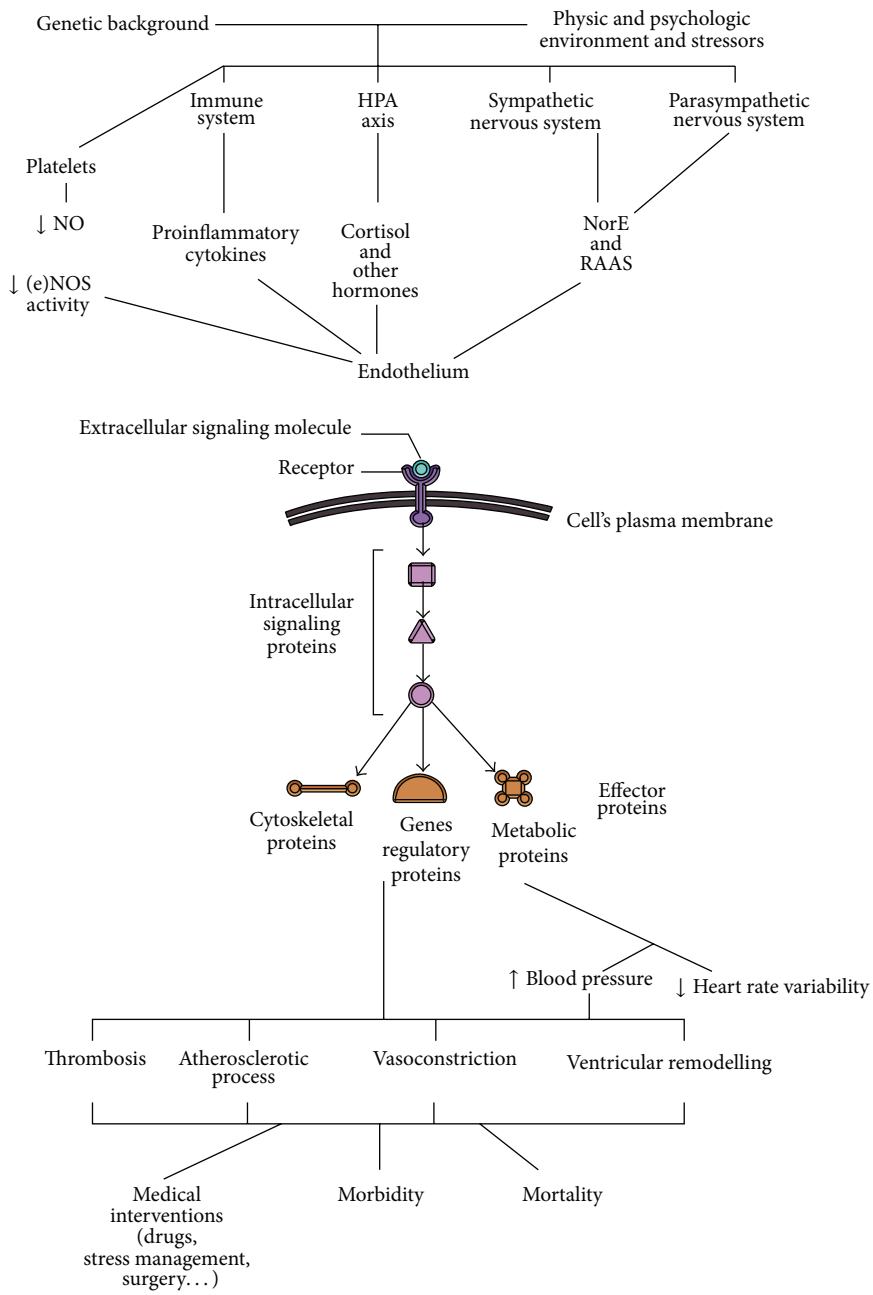

Figure 2: "Internal" and "external" stimuli activate and balance endocrine, immune, and psychoneurological responses. Cells, in the figure of endothelial cells, interact with the surrounding environment by interpreting extracellular signals via receptor proteins that span their membrane. Receptors are composed of extracellular and intracellular domain. The inner domain interacts with other intracellular signaling proteins that relay the message to one or more effector proteins. These proteins activate gene transcription and mediate different responses. (HPA axis: hypothalamus-pituitary-adrenal axis; NO: nitric oxide; (e)NOS: nitric oxide synthesis; NE norepinephrine; RAAS: renin-angiotensin-aldosterone system).

the cells of the adrenal zona glomerulosa after binding to the PTH/PTH-rP receptor and indirectly by potentiating angiotensin-2 induced effects. This may explain why parathyroidectomy decreases aldosterone levels in parallel with improved cardiovascular outcomes. Aldosterone mediated effects are inappropriately pronounced in conditions such as chronic heart failure, excess dietary salt intake (relative aldosterone excess), and primary aldosteronism [37]. PTH is increased as a result of (1) the mineralocorticoid receptor (MR) mediated calciuretic and magnesiuretic effects and (2) direct effects of aldosterone on parathyroid cells via binding to the MR. Hyperparathyroidism causes myocardial fibrosis and disturbed bone metabolism. Furthermore, a link between Vitamin D, renin-angiotensin-aldosterone system, and the fibroblast growth factor 23/klotho pathways has been discovered recently and highlighted as an active cardiovascular

regulator system [38, 39]. Hyperaldosteronism due to klotho deficiency results in vascular calcification, which can be mitigated by treatment with the aldosterone receptor antagonist spironolactone [40].

In asymptomatic primary hyperparathyroidism (PHPT), CFR was assessed as a marker of coronary microvascular function and inversely related to PTH levels. PTH independently correlated with the coronary microvascular impairment, suggesting a crucial role for the hormone to explain the increased cardiovascular risk in PHPT. Furthermore, coronary microvascular dysfunction in PHPT patients was completely restored after parathyroidectomy [21].

2.2. Thyroid Hormones. There is an association between subclinical hypothyroidism (SCH) and increased risk for cardiovascular disease as shown in a study performed by Traub-Weidinger et al. examining coronary vascular reactivity in asymptomatic patients with SCH before and after levothyroxine (LT4) supplementation [41]. In asymptomatic subjects with SCH due to thyroid autoimmunity, coronary microvascular function was impaired and improved after supplementation with LT4. These findings may explain the increased cardiovascular risk attributed to SCH [41].

Savinova et al. demonstrated that low thyroid hormone (TH) function influences coronary remodeling and reduces the density of small arterioles in heart failure. In fact, hypothyroidism results in arteriolar atrophy in the left ventricle. In this context, tri-iodothyronine (T3) treatment rapidly induces small arteriolar muscularization and, within 72 hours, restores arteriolar density to normal levels. T3 treatment results in the coordinate regulation of angiopoietin 1 and 2 expression. The response to angiopoietins leads to vessel enlargement. In addition to the effects of THs on vasoreactivity, these results suggest that THs may affect the function of small resistance arteries also influencing the remodeling of vascular smooth muscle cells [42].

Finally, TH is critical for living organisms when coping with environmental stress. Plasma circulating T3 levels drop in most disease states and are associated with increased oxidative stress [43, 44]. In this context, plasmatic T3 levels are an independent determinant for the recovery of cardiac function in patients after myocardial infarction [45]. Thyroid hormone receptor α1 (TRα1) seems to be crucial in this response. TRα1 translocates into the cell nucleus upon activation of stress induced growth kinase signaling. Furthermore, overexpression of nuclear TRα1 in cardiomyocytes can result in pathological or physiological growth (dual action) in absence or presence of its ligand, respectively. Accordingly, inactivation of TRα1 receptor preventing the reactive hypertrophy following myocardial infarction results in heart failure with increased phospholamban (PLB) expression and marked activation of p38MAPK [45]. In line with this evidence, TH limits ischemia/reperfusion injury and converts pathologic to physiologic growth after myocardial infarction via TRα1 receptor. In this view, TRα1 receptor may become a novel pharmacological target for cardiac repair/regeneration therapies [45].

2.3. Growth Hormone and Insulin Like Growth Factor-I. Growth hormone (GH) and insulin like growth factor-I (IGF-I) affect cardiac structure and performance. In the general population, low IGF-I has been associated with higher prevalence of ischaemic heart disease and mortality. Recent epidemiological evidence suggests that serum IGF-I levels in the low-normal range are associated with increased risk of acute myocardial infarction, ischaemic heart disease, coronary and carotid artery atherosclerosis, and stroke [46]. This confirms previous findings in patients with acromegaly or with GH-deficiency showing cardiovascular impairment [47]. Patients with either childhood- or adulthood-onset GHD have cardiovascular abnormalities such as reduced cardiac mass, impaired diastolic filling and left ventricular response at peak exercise, increased intima-media thickness, and endothelial dysfunction. These abnormalities can be reversed, at least partially, after GH replacement therapy [48].

CFR has been shown to decrease in adults with GHD. Direct correlation between CFR and IGF-1 concentrations suggests that GH replacement may improve microvascular function and likely decrease cardiovascular morbidity and mortality in patients affected by GHD [49].

On the contrary, in acromegaly, chronic GH and IGF-I excess causes a specific cardiomyopathy, that is, concentric cardiac hypertrophy (diagnosed in more than two-thirds of the patients) associated with diastolic dysfunction [47]. In later stages, impaired systolic function and heart failure can occur if GH/IGF-I excess is not controlled. Abnormalities of cardiac rhythm and of heart valves are also reported. Successful medical or surgical control of acromegaly is accompanied by the decrease of the left ventricular mass and improvement of cardiac function [48].

2.4. Cortisol. Coronary microvascular function, as assessed by CFR, is pathologically reduced in a considerable number of patients with Cushing's syndrome without clinical symptoms of ischemic heart disease and in the absence of epicardial coronary artery lesions [50]. Although the presence of comorbidities has to be taken into account to explain this early coronary abnormality in Cushing patients, CFR inversely relates to urinary cortisol in patients with endogenous hypercortisolism. Nevertheless, the possibility of using urinary cortisol as a predictor of coronary microvascular function in patients with Cushing's syndrome needs further investigation [50].

2.5. Sexual Hormones. Sexual hormones affect endothelial function. The protective effects of estrogens on cardiovascular function are well known. Females in their premenopausal period of life present a lower incidence of cardiovascular events in comparison with men of the same age and risk factor profile [51]. In postmenopause this difference tends to disappear. Estrogen administration in postmenopausal women is associated with a 50% reduction in the clinical manifestations of coronary artery disease [52]. One explanation seems to be the estrogen-induced modulation of coronary vasoreactivity. In fact, estrogens decrease basal coronary vasomotor tone as manifested by increased coronary flow,

decreased resistance, and increased epicardial cross-sectional area. These hormonal actions on coronary vasoreactivity may explain, in part, the cardioprotective effects of estrogen in postmenopausal women [51, 53].

Epidemiological studies have shown a high prevalence of low serum testosterone levels in men with cardiovascular disease. Furthermore, low testosterone levels are associated in some, but not in all observational studies, with an increase in cardiovascular events and mortality [54]. Testosterone has beneficial effects on several cardiovascular risk factors, which includes cholesterol, endothelial dysfunction, and inflammation. Testosterone has vasodilatory actions on several vascular beds, although some studies have reported conflicting effects [54]. In clinical studies, acute and chronic testosterone administration increases coronary artery diameter and flow, improves cardiac ischemia and symptoms in men with chronic stable angina, and reduces peripheral vascular resistance in chronic heart failure. Testosterone is an L-calcium channel blocker and induces potassium channel activation in vascular smooth muscle cells. Animal studies [54] have consistently demonstrated that testosterone is atheroprotective, whereas testosterone deficiency promotes the early stages of atherogenesis; however, the mechanisms involved are not completely understood.

The role of testosterone on vascular function has been investigated also in heart transplanted patients. Among the various complications of heart transplantation (HTx), the vasculopathy of the allograft (CAV) represents a serious problem linked to chronic rejection [55]. Testosterone plasma levels may be involved in CAV development indirectly, increasing traditional risk factors and directly influencing the alloimmune response [55]. These findings have been confirmed assessing vascular erectile dysfunction (ED) and CFR in a group of heart transplanted patients. CFR was significantly reduced in ED versus no-ED patients and lower testosterone plasma levels were statistically associated with CAV [56]. These data about the effects of testosterone on endothelial function are in line with another study performed in a group of women with polycystic ovary syndrome (PCOS). Total testosterone, free testosterone, and androstenedione were increased in case of PCOS and CFR was preserved in this clinical condition [57].

Two recent studies raised some concerns about cardiovascular risks associated with testosterone therapy [58, 59]. Morgentaler et al. concluded that so far the available evidence of an increased cardiovascular risks induced by testosterone therapy is rather inconclusive and needs further appraisal [60].

2.6. Insulin and Glucagon-Like Peptide-1. Insulin has an important role in vascular function and recent evidence documents insulin-mediated vasodilatory effects at the level of coronary vessels [61]. In healthy subjects insulin enhances myocardial blood flow and decreases coronary vascular resistance in a dose-dependent manner [62]. Moreover, insulin is able to increase myocardial blood flow also in conditions characterized by coronary dysfunction such as obesity, diabetes type 1, and coronary artery disease. In contrast, hyperinsulinemia and insulin resistance, which are associated with an impaired vasodilatory effect of insulin, have been demonstrated to be an independent risk factor for coronary artery disease [63]. Hyperglycemia after oral glucose loading suppresses CFR in healthy young male subjects [64]. This result suggests that acute hyperglycemia may have adverse effects on coronary microcirculation. Instead, control or improvement of hyperglycemia improves CFR, as shown in poorly controlled diabetic patients [65].

Glucagon-like peptide-1 (GLP-1) has protective effects in the heart [66], mitigating coronary microvascular dysfunction through a reduction in oxidative stress [67]. The protective effects of GLP-1 are dependent on downstream inhibition of Rho through a cAMP/PKA-mediated pathway [68]. Moreover, GLP-1 regulates glucose-dependent insulin secretion and has numerous extrapancreatic effects, including the recruitment of cardiac muscle microvasculature in healthy humans [69].

2.7. Obesity. Obese patients have reduced myocardial vasoreactivity, which may represent an early precursor of future coronary artery disease. Insulin-induced enhancement of myocardial blood flow is blunted in obesity [70] but also serum leptin, a hormone produced by adipose tissue, is inversely related to the adenosine-stimulated myocardial flow suggesting a vasoactive role [71]. The early coronary microvascular impairment of obesity seems also to be related to a systemic chronic inflammation mediated by adipocytokines, independently of body max index [72]. Body weight loss improves coronary circulatory dysfunction [73] and bariatric surgery rapidly reverses obesity-induced endothelial dysfunction [74, 75] via a GLP-1-mediated mechanism [76].

2.8. Adipose Tissue and Adipokines. Fat cells surround coronary arteries and play a central although underrecognized role in the development of cardiovascular disease through the systemic secretion of adipokines. Adipokines are protein produced by the adipose tissue (especially by periadventitial adipose tissue) with paracrine and endocrine actions [77] able to regulate inflammatory molecule expression [78]. Spiroglou et al. showed that vascular dysfunction and atherosclerotic lesions are positively correlated with chemerin, vaspin, visfatin, and leptin fat expression at periaortic level [79]. Furthermore, coronary atherosclerosis positively correlates with chemerin and visfatin pericoronary fat expression and coronary flow impairment with chemerin and vaspin expression [80].

2.9. Vasopressin and Oxytocin. Vasopressin (VP) and oxytocin (OT) are mainly synthesized in the magnocellular neurons of the paraventricular and supraoptic nucleus of the hypothalamus and released into the blood from their axon projections in neurohypophysis. VP and OT act in complementary manner in cardiovascular control, as both hormones and neurotransmitters, regulating Ca^{2+} signaling [81]. While VP conserves water and increases circulating blood volume, OT eliminates sodium [82]. In most vascular beds VP is a potent vasoconstrictor [83], more potent than OT.

The vasoconstriction by VP and OT is mediated via V1a receptor [84, 85]. Instead, in some vascular beds, such as the lungs and the brain, VP and OT produce NO dependent vasodilatation [86]. Martínez et al. studied the coronary effects of VP and its interaction with NO and prostanoids in a goat model, during partial ischemia and reperfusion. Ischemia led to the reduction of coronary vasodilatory reserve and attenuation of VP-induced vasoconstriction; the modulatory role of NO was preserved and there was a probable involvement of vasoconstrictor prostanoids. During reperfusion, the coronary vasodilatory reserve and the coronary reactivity to acetylcholine and VP are recovered [87].

Peripherally, VP has been found to enhance the sensitivity of the baroreceptor; instead centrally, VP and OT increase sympathetic outflow, suppress baroreceptor reflex, and enhance respiration [85]. While VP is an important mediator of stress followed by adrenocorticotropic hormone (ACTH) release, OT exhibits antistress properties [88]. Moreover, VP has been found to contribute considerably to the progression of hypertension and heart failure while cardiovascular actions of OT include lowering blood pressure, negative inotropic and chronotropic effects, parasympathetic neuromodulation, vasodilatation, anti-inflammatory activity, antioxidant activity, and metabolic effects [88]. OT actions are mediated by NO and ANP [89]. Recent evidence suggests that the enhanced stimulation of central angiotensin-1 and V1 receptors as well as the attenuated stimulation of oxytocin receptors accounts for the exaggerated cardiovascular responses to stress stimuli during the postinfarct state and that, on the contrary, angiotensin II, vasopressin, interleukin-1, and tumor necrosis factor-α (TNF-α) systems are important in the central cardiovascular control under resting conditions [90, 91]. In experimentally induced myocardial infarction, continuous *in vivo* OT delivery improves cardiac healing and cardiac work, reduces inflammation, and stimulates angiogenesis [86, 88, 92].

2.10. Prolactin. Recent studies show that hyperprolactinemia is associated with endothelial dysfunction, increased carotid intimal medial thickness, insulin resistance, and low-grade inflammation [93]. On the other hand, prolactin has been suggested to play an autocrine regulatory role in angiogenesis induced by the FGF2/STAT5 signaling cascade and VEGF expression induction [94].

Moreover, the treatment of porcine aortic endothelial cells by prolactin caused a reduction of NO production causing coronary, mesenteric, renal, and iliac vasoconstriction [95]. The stress hormone prolactin could be a costimulator of platelet activation in patients with acute coronary syndrome [96]. A pilot study showed that patients with prolactinoma are characterized by microvascular dysfunction as well as plasma markers indicating a proatherothrombotic state [97]. Prolactin is closely associated with autoimmune diseases in animal models and humans, and several disease-related autoantibodies were increased in patients with hyperprolactinemia [98]. Interestingly, the presence of antiendothelial cell antibodies may explain the prolactin's proinflammatory and proatherothrombotic effects [98].

The role of prolactin in vascular and myocardial regulation may be different according to the site of action and needs therefore to be better clarified.

2.11. Melatonin. Melatonin, the principal hormone of the vertebral pineal gland, exerts endothelial-dependent vasorelaxant effects, which potentiate significantly the effect of acetylcholine, and counteracts the vasoconstrictor responses to catecholamines [99]. These effects may be, in part, due to a melatonin favourable influence on the redox balance, with elevated NO and cGMP levels along with lower calcium in vascular tissue [100]. In animal models, melatonin decreases also the inflammatory factors acting on endothelial cells [101] and preserves capillary perfusion during ischemia-reperfusion events [102].

2.12. The Liver: Bilirubin, Heme Catabolic Pathway, and γ-Glutamyltransferase. Recent data has convincingly demonstrated that mildly elevated serum bilirubin levels are strongly associated with a lower prevalence of oxidative stress-mediated diseases [103]. Indeed, serum bilirubin has been shown to negatively correlate to CV diseases, as well as to CV risk factors such as arterial hypertension, diabetes mellitus, metabolic syndrome, and obesity. This data suggests a protective effect of bilirubin and of other products of the heme catabolic pathway such as biliverdin and carbon monoxide, as well as the key enzymes, heme oxygenase, and biliverdin reductase [103]. The heme-heme oxygenase system has recently been recognized to possess important regulatory properties: it is tightly involved in both physiological and pathophysiological processes, such as cytoprotection, apoptosis, and inflammation. Effects of the free heme on the vascular system are determined by extracellular factors, such as hemoglobin/heme-binding proteins, haptoglobin, albumin, hemopexin, and intracellular factors, including heme oxygenases and ferritin [104].

Serum γ-glutamyltransferase (GGT) levels are an independent risk factor for CV disease, and there is a strong association between serum GGT levels and most CV risk factors. In fact, high serum GGT levels, which correlate with low CFR, represent an independent marker of coronary microvascular damage and inflammation in normal individuals without concomitant risk factors [105].

3. Beyond CFR

3.1. Inflammation and Immune Function. Atherosclerosis has been identified as an inflammatory process [106] driven by the adaptive (T and B cells) immune system and dendritic cells [107]. Cardiac patients with increased levels of proinflammatory cytokines, such as TNF-α and IL-6, have increased risk of adverse clinical events [36]. Increased levels of proinflammatory cytokines are also found in heart failure patients likely as the consequence of cardiac remodeling [36]. We also documented a CFR reduction in young patients with severe psoriasis without coronary disease, suggesting that coronary microvascular dysfunction was an early complication of psoriasis independently related to

the severity and extension of the skin manifestations and likely the consequence of the chronic systemic inflammation [108].

3.2. Platelet Function. Platelet abnormalities, such as increased activation or disturbed serotonin metabolism, causing platelet hyperreactivity and thrombosis, are linked to higher cardiovascular morbidity and mortality [36]. Even though myeloproliferative neoplasms are most commonly associated with venous thrombosis, up to 60% of patients experience a thrombotic event in their lifetimes, including stroke or myocardial infarction. We documented CFR reduction in asymptomatic patients with essential thrombocythemia and polycythemia vera [109]. These patients have coronary microvascular dysfunction in absence of clinical conditions suggesting CAD. Sickle cell disease is characterized by obstruction of microvessels leading to ischemia and necrosis and in this clinical setting an abnormal cardiac perfusion reserve is present [110]. Vasoocclusion represents a phenomenon involving endothelial cell dysfunction, leukocyte activation, platelet activation, and chronic inflammation resulting in multiple adhesive interactions between these cellular elements [111]. Since platelets mediate inflammation as well as thrombosis via release of pro- and anti-inflammatory molecules, they are crucial players to maintain cardiovascular health and to balance different neuroendocrine-immune signals [109].

3.3. Autonomic Balance. Stress, anxiety, and depression contribute to cardiovascular diseases including heart failure, ischemic disease, hypertension, and arrhythmias [112]. The appropriate balance (autonomic tone) between the sympathetic and the parasympathetic system, which are the two major components of the autonomic nervous system, is fundamental in the pathophysiology of cardiovascular diseases [112]. Chronic activation of the sympathetic system (which occurs in cases of chronic stress, depression, or anxiety, including also personality disorders) and/or decreased parasympathetic (vagal) tone is a hallmark of cardiovascular disease. The sympathetic system contributes to endothelial dysfunction, hypertension, and atherosclerosis [113, 114]. It promotes insulin resistance and dyslipidemia [115, 116] but also induces left ventricular hypertrophy [117], increases the incidence of arrhythmia, and promotes renal dysfunction by stimulating sodium and fluid retention [118], glomerulosclerosis, and the activation of the renin-angiotensin-aldosterone system (RAAS) [112, 119]. Chronic stress increases vascular responses to noradrenaline. This effect is endothelium-dependent and involves the release of vasoconstrictor prostanoids via stimulation of endothelial alpha-2 adrenoceptors [120]. Several lines of evidence support the role of inflammation and immune mechanisms in the pathogenesis of heart diseases [121] and endothelial dysfunction [122]. Interestingly, the nervous system can directly activate the immune system [123] and an inflammatory process may arise after the release of neuropeptides from nerves, in a process called "neurogenic inflammation" [124].

4. The Stress Response

Hans Selye's [125] inspired a huge and still growing wave of medical research. His experiments with rats led to the recognition of the "general adaptation syndrome," later renamed by Selye "stress response": the triad of enlarged adrenal glands, lymph node and thymic atrophy, and gastric erosions/ulcers [126].

All organisms maintain a complex dynamic equilibrium, called homeostasis, which is constantly challenged by internal or external adverse forces termed "stressors" (hotness, coldness, toxins, infections, wounds, fatigue, psychosocial factors, etc.) [127]. Stress occurs when homeostasis is threatened or perceived to be so; homeostasis is reestablished by various physiological and behavioral adaptive responses that constitute the so-called "stress response" [128]. Thus stress could be defined, according to the original Selye's definition, as the general and nonspecific response to any request from the environment. Under favorable conditions, individuals can develop vegetative and pleasurable responses that enhance their emotional and intellectual growth and help the survival of their species, such as food intake and sex [129]. In contrast, activation of the stress response during threatening situations beyond the control of the individual can be associated with dysphoria and eventually emotional or somatic disease [130]. Tsigos and Chrousos reviewed the mechanisms underlying the stress response [130] (Figure 3). Briefly, "the main components of the stress system are the corticotropin-releasing hormone (CRH) and locus ceruleus-norepinephrine- (LC/NE-) autonomic systems and their peripheral effectors, the pituitary-adrenal axis, and the limbs of the autonomic system. An active stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances for survival. The CRH and LC/NE systems stimulate arousal and attention, as well as the mesocorticolimbic dopaminergic system, which is involved in anticipatory and reward phenomena, and the hypothalamic beta-endorphin system, which suppresses pain sensation and, hence, increases analgesia. CRH inhibits appetite and activates thermogenesis via the catecholaminergic system. Moreover, reciprocal interactions exist between the amygdala and the hippocampus and the stress system, which stimulates these elements and is regulated by them. During stress CRH inhibits GnRH and, through somatostatin, GH, TRH, and TSH secretion, which in turn, suppress the reproductive, growth, and thyroid functions. Interestingly, all these functions receive and depend on positive catecholaminergic input. The hormones at the end of the hypothalamic-pituitary-adrenal (HPA) axis and glucocorticoids have multiple roles. They simultaneously inhibit the CRH, LC/NE, and b-endorphin systems and stimulate the mesocorticolimbic dopaminergic system and the CRH peptidergic central nucleus of the amygdala. In addition, they directly inhibit pituitary gonadotropin, GH, and TSH secretion, render the target tissues of sex steroids and growth factors resistant to these substances, and suppress the 50 deiodinase, which converts the relatively inactive tetraiodothyronine (T4) to triiodothyronine (T3), contributing further to the suppression of reproductive,

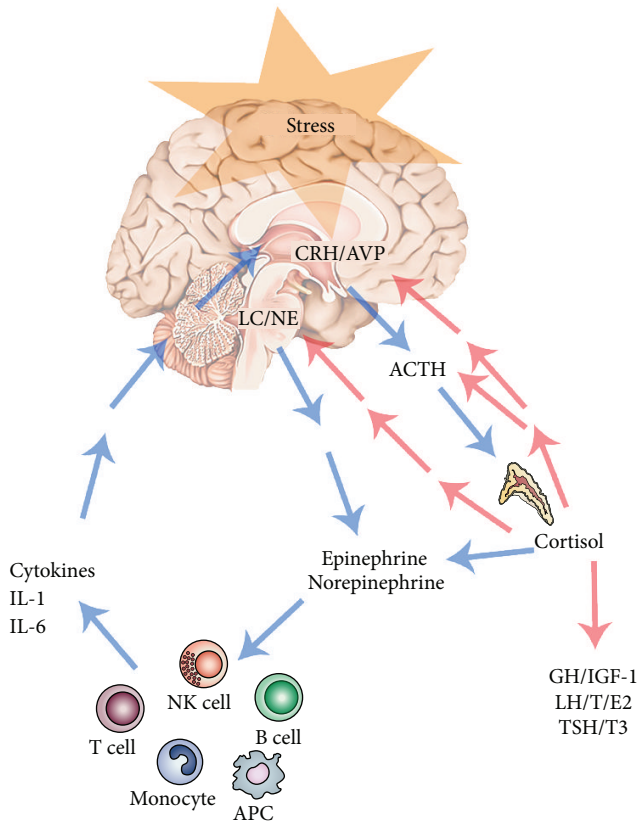

Figure 3: A simplified schematic representation of the stress system and the effectors of the stress response. The CRH/AVP neurons and central catecholaminergic neurons of the LC/NE system are reciprocally connected. Several feedback loops control the time-integrated secretion of cortisol and the activity of the HPA axis. Furthermore glucocorticoids stimulate the fear centers in the amygdala. Activation of the HPA axis leads to the suppression of the GH/IGF-1, LH/testosterone (T)/estradiol 2 (E2), and TSH/T3 axes. HPA axis, sympathetic system, and immune system activity are related. Blue lines indicate stimulation; red lines indicate inhibition. Abbreviations are as in the text. Modified from [130].

growth, and thyroid functions. They also have direct as well as insulin-mediated effects on adipose tissue, ultimately promoting visceral adiposity, insulin resistance, dyslipidemia and hypertension (metabolic syndrome X), and direct effects on the bone, causing "low turnover" osteoporosis. Central CRH, via glucocorticoids and catecholamines, inhibits the inflammatory reaction, while directly secreted by peripheral nerves CRH stimulates local inflammation [124] (immune CRH)" [130].

As demonstrated by Charmandari et al., appropriate responsiveness of the stress system to stressors is a crucial prerequisite for a sense of wellbeing, adequate performance of tasks, and positive social interactions. By contrast, inappropriate responsiveness of the stress system may impair growth and development and may account for a number of endocrine, metabolic, autoimmune, and psychiatric disorders [131, 132]. The development and severity of these conditions primarily depend on the genetic vulnerability of the individual, the exposure to adverse environmental factors, and the timing of the stressful events, given that prenatal life, infancy, childhood, and adolescence are critical periods characterized by increased vulnerability to stressors [131, 133]. A hyper- or hypoactive stress system associated with abnormalities of the systemic anti-inflammatory feedback and/or hyperactivity of the local proinflammatory factors play a relevant role in the pathogenesis of chronic inflammation and immune-related diseases, such as atherosclerosis, hypertension ischaemic heart diseases, or heart failure [132].

4.1. Mental Stress Ischaemia. There is a great amount of literature on psychological stress (see "stress response" below) and cardiovascular disease [134]. Studies about the effects of acute stressors have been performed in people that experienced disasters (earthquakes or hurricanes) [135, 136] while studies about chronic stressors evaluated, for example, the effects of job stress [137], marital unhappiness [138, 139], and burden of caregiving [140]. From all of these studies there are extensive data concerning stressors' contributions to diverse pathophysiological changes including sudden death, myocardial infarction, myocardial ischemia, and wall motion abnormalities, as well as to alterations in cardiac regulation as indexed by changes in sympathetic nervous system activity and hemostasis [140]. The concept of "personality" is intimately linked to the concept of mental stress. Some personality patterns [141], that is, depressive or aggressive moods, are linked with higher incidence of cardiovascular

diseases through chronic stress axis activation [36, 142]. Mostofsky et al. described outbursts of anger as a trigger of acute cardiovascular events [143]. Prolonged impairment of endothelial function has been documented in healthy men after a brief episode of mental stress [144, 145] and CFR reduction [146, 147]. The Takotsubo cardiomyopathy (or "stress cardiomyopathy") consists of a transient left ventricular dysfunction triggered by acute emotional or physical stress, whose clinical presentation mimics acute myocardial infarction, with acute chest pain, transient ST elevation, and apical ballooning on echocardiography. Although its cause remains elusive, coronary artery vasospasm, coronary microcirculation dysfunction [148], obstruction of the left ventricular outflow tract (LVOT), and catecholamine overload have been proposed as mechanisms for the injury. Myocytolysis and the histopathological lesions observed in takotsubo cardiomyopathy have been related with several brain conditions and neurogenic mechanisms of cardiac disease like catecholamine infusion, brain stimulation, and stress. Interestingly, evidence shows that heart lesions may occur even in adrenalectomized animals (although less pronounced), which suggests that these findings may be dependent on the direct action of nerve terminals in the heart [112]. All these considerations may explain the lack of efficacy of beta-blockade on low heart rate-related ischemia during mental stress [149] and highlight the importance of stress management in prevention and treatment of cardiovascular diseases [140].

4.2. Cellular Signaling Pathways. Generally, cells involved in regulating CV homeostasis respond to changes in their local environment using a range of receptors, among which the G-proteins coupled receptors are the most important. A signal recognition is then transformed into a cellular response (physiological or pathological) through intracellular transduction mechanisms that converge on the regulation of the phosphorylation state of intracellular proteins by a range of protein kinase and protein phosphatase enzymes (Figure 2). It seems likely that subtle defects in these mechanisms may lead to a number of cardiovascular pathologies. The high complexity of these signaling systems allows various cells to act in concert to maintain homeostasis responding rapidly to small and fluctuating changes in the incoming environmental signals, while the crosstalk between signaling pathways allows coordinated responses to multiple different and sometimes opposing signals [150, 151]. As an example, the receptor for the epidermal growth factor (EGF) and related ligands (EGFR), the prototypal member of the superfamily of receptors with intrinsic tyrosine kinase activity, is widely expressed on many cell types, including epithelial and mesenchymal lineages [152]. Upon stimulation by at least five genetically distinct ligands (including EGF, transforming growth factor-α (TGF-α), and heparin-binding EGF (HB-EGF)), the intrinsic kinase is activated and EGFR tyrosyl phosphorylates itself and numerous intermediary effector molecules, including closely related c-erbB receptor family members [153]. This step initiates multiple signaling pathways, some of which are involved in negative feedback loops [154]. The integrated biological responses to EGFR signaling are pleiotropic including mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation [155]. EGF network has a critical role in normal heart function and in normal cardiac valve formation in conjunction with ErbB receptors [156]. Moreover, current evidence suggests that angiotensin II receptor mediates transactivation of the EGF receptor on cardiac myocytes involving stimulation of the activities of a family of membrane-associated metalloprotease enzymes [150]. These enzymes ultimately cleave EGF receptor ligands (such as heparin-binding EGF) from their membrane-associated precursors; once released these ligands can stimulate EGF receptors leading to the activation of several signaling pathways in the myocytes [150]. Phosphatase and kinase activities regulate a number of cytosolic and nuclear phosphorylation events which together control the myocardial gene transcription involved in ventricular hypertrophy [150].

Understanding which mechanisms activated by extracellular stimuli are able to modify the cardiac and vascular cell functions will allow a deeper insight into the pathophysiology of cardiovascular disease. This knowledge will lead to the identification of novel molecular targets for pharmacological intervention and will assist the future development of therapeutic strategies against cardiovascular disorders.

5. The Heart and Cardiovascular System in the "Psychoneuroendocrine Immunologic" Network

Psychoneuroendocrine immunology studies the interactions among behavioral, neural and endocrine, and immunologic processes of adaptation [157]. The exploration of the extensive interactions among psychological and behavioral factors, the nervous system, the immune system, and the endocrine system may help understand the mechanisms underlying health, wellness, and diseases [158]. Many studies evidences have supported the close relationships between stress, depression, inflammation, and disorders including diabetes, obesity, and cardiovascular disease [159].

Psychological and nervous factors act on the cardiovascular system. The immune system is primarily involved in the pathological processes leading to left ventricular dysfunction and fibrosis [160] and communicates with the nervous and endocrine systems to maintain cardiac homeostasis [147].

The hormonal influence on the heart extends beyond endothelial function. For example, both cardiac myocytes and cardiac stem cells express the growth hormone releasing hormone receptor (GHRH), whose activation improves injury responses after myocardial infarction, reversing ventricular remodeling and enhancing heart functional recovery [161]. We already discussed the action of T3 on cardiomyocytes via TRα1. Furthermore, parathyroid hormone improves contractile performance of adult rat ventricular cardiomyocytes at low concentrations [162]. Growth factors that stimulate proliferation of fetal cardiomyocytes include angiotensin II, cortisol, and IGF-1. Two normally circulating

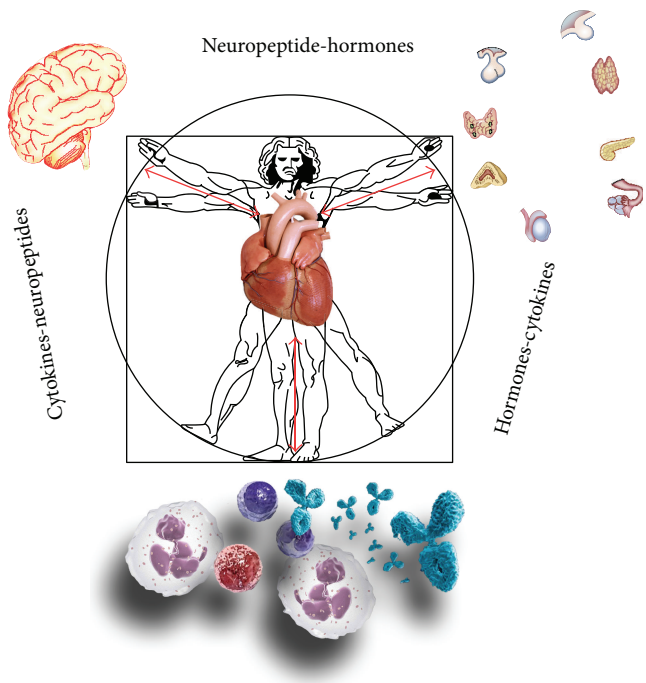

Figure 4: The cardiovascular system in the psychoneurological, hormonal, and immune network: it receives and sends signals to the brain, to the immune, and to the endocrine system [75]. As in Leonardo's Vitruvian man, also this network represents harmony and perfection in proportions. Psyche, brain and nerves, glands and hormones, lymphocytes, and cytokines are synchronized, they interact with each other, and the harmony between these systems involves and regulates cardiovascular functions.

hormones, atrial natriuretic peptide and T3, suppress cardiomyocytes proliferation [163].

Furthermore, the heart acts as a gland, for example, secreting atrial natriuretic peptide, and as an immune relay [164] which gives afferent neurological input to the brain [165, 166]. There is dynamic, bidirectional communication between the heart and brain influencing reciprocal functions. The heart communicates with the brain in four major ways: neurologically (through the transmission of nerve impulses) and electrically [167], biochemically (via hormones and neurotransmitters), and biophysically (through pressure waves).

6. Conclusion

In the present review, we depicted how psychoneurological, hormonal, and immune functions affect and regulate cardiovascular homeostasis and in particular coronary function (Figure 4). Nowadays, the cardiovascular system is conceived as the centre of a complex multiple organ network in which all components contribute to our health and wellbeing [168]. In this view, integrative medicine has emerged as a new therapeutic model that is patient centered and healing oriented [169]. Such patient care emphasizes the therapeutic relationship and uses therapeutic approaches originating from both conventional medicine and alternative medicine, such as meditation [170–172], music listening [173, 174], alimentation [175, 176], or physical exercises [177, 178]. All these lifestyle and behavioral aspects, counteracting the stress response, have a positive effect on our health and on our cardiovascular system [179]. In this context, the regulation of coronary reserve function clearly shows the importance of psychoneuroendocrine-immunitary factors in physiological but also pathological conditions and suggests the need to explore new therapeutic horizons against coronary artery disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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