The Cardiovascular Effects of Chronic Hypoestrogenism in Amenorrheic Athletes: A Critical Review

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Abstract

In premenopausal women, the most severe menstrual dysfunction is amenorrhea and is associated with chronic hypoestrogenism. In postmenopausal women, the coincident hypoestrogenism observed is associated with a number of clinical sequelae related to cardiovascular health. Due to an observed cardioprotective effect of E2, persistently low E2 levels in amenorrheic (AM) athletes may confer deleterious effects on cardiovascular health. The incidence of amenorrhea among athletes is much greater than that observed among sedentary women. Recent data in AM athletes demonstrate impaired endothelial function, elevated low- and high- density lipoprotein levels, reduced circulating nitrates and nitrites, and increased susceptibility to lipid peroxidation. An improvement in endothelial function has also been reported during the recovery of menstrual cyclicity in a small sample of previously AM athletes. Although no longitudinal studies exist, these findings are suggestive of increased risk of premature cardiovascular disease in AM athletes. These issues are explored and discussed in detail in this comprehensive review paper. Future research should focus on the presentation and progression of these adverse cardiovascular parameters in physically active women and athletes with hypoestrogenism to determine their effect on long-term health outcomes.

Key words: amenorrhea, athlete, endothelium, c-reactive protein, endothelin, E2, homocysteine, lipids, nitric oxide, exercise.

Introduction

Unprecedented numbers of women are now participating in physical activity and sport on a regular basis [1]. The physiologic benefits of regular exercise are well documented. However, between 2 and 46% of athletic women have reported experiencing amenorrhea (absence of menstruation for 3 consecutive months or greater) at any given time, compared to 2-5% of eumenorrheic sedentary women [2]. A chronic deficit in energy intake relative to energy expenditure is likely the primary cause of exercise-associated amenorrhea [3, 4, 5], although insufficient oxidizable metabolic fuel [6], and psychological stress [7] have also been indicated.

The harmful effects of hypoestrogenism upon bone health in amenorrheic (AM) athletes has previously been reported [8, 9]. Specifically, a medical condition termed The Female Athlete Triad defines the co-existence of disordered eating, amenorrhea and low bone mass in athletes [2, 10]. Since a cardiovascular role for E2 has been identified, impaired cardiovascular health has been suggested as an additional clinical sequelae of hypoestrogenism [11]. For example, it has been demonstrated that chronically hypoestrogenism exerts unfavorable effects upon serum lipids [12, 13], vascular tone [14], hemostatic parameters [15], blood flow [16], homocysteine [17, 18], and antioxidant status [19]. However, much of these data derives predominantly from studies of postmenopausal women, as well as animal and in vitro models.

In AM athletes, recent data identifies impaired endothelial function [11], elevated low-density and high-density lipoprotein levels [20], reduced metabolites of nitric oxide, (nitrates and nitrites) [21], and increased susceptibility to lipid peroxidation [22] as potential cardiovascular consequences, presumably due to the hypoestrogenism. Although the clinical significance of these consequences remains undetermined, these findings are suggestive of a potentially increased risk of premature cardiovascular disease (CVD).

This purpose of this paper is to bring together the relevant data surrounding exercise-associated amenorrhea using both traditional and novel markers of CVD. Due to the dearth of information on AM athletes and cardiovascular health, data derived from postmenopausal women, animal models, and in vitro studies are drawn upon to provide an indication of how E2 status may impact such markers. While these studies offer insights to the effects of the hypoE2ic milieu, there are obvious limitations when extrapolating these findings to AM athletes, i.e., the postmenopausal woman is likely to present with multiple risk factors that are inherent with aging, whereas the AM athlete will likely have minimal or no risk factors. Thus, extrapolations are intended simply to assist our ability to understand the effects of hypoestrogenism *per se* on key cardiovascular health parameters. We will also utilize comparisons to anorexic women due to similarities in age, menstrual status, and often, exercise behaviors. Serum lipids, lipid peroxidation, nitric oxide, and endothelin are examined in this review. Due to the predictive value of recently identified novel markers to assess risk of future cardiovascular disease or events, endothelial function, homocysteine and c-reactive protein are also discussed.

1. Total Cholesterol

1.1 Introductory Comments: Cholesterol, a waxy, fat like substance, is present in every cell in the body. Functionally, cholesterol is important to cellular membrane structure, as well as to synthesize vitamin D, the adrenal gland hormones, and the steroid hormones, namely E2, progesterone, and androgens [23]. Cholesterol also plays a key role in the formation of the bile secretions that emulsify fat during digestion [23]. Cholesterol is derived from dietary sources and is synthesized de novo, predominantly by the liver and intestines, in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA [23]. The level of cholesterol

synthesis is regulated, in part, by 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, the rate-limiting enzyme in cholesterol biosynthesis [24]. Dietary fat intake, specifically saturated fatty acids, also regulates cholesterol levels by increasing the hepatic sterol pool and by down regulating LDL receptors, the principle route of clearance of LDL cholesterol [25], the primary cholesterol-containing atherogenic lipoprotein (60-70% cholesterol) [26]. The other two major classes of lipoprotein particles found in fasted serum are (1) high density lipoproteins (HDL), considered the atheroprotective lipoprotein (20-30% cholesterol), and (2) very low density lipoproteins (VLDL), primarily a triglyceride-containing lipoprotein (10-15% cholesterol) [26].

The link between blood lipid levels and CVD is well recognized [27,26]. Desirable serum total cholesterol (TC) levels are identified as <5.18 mmol/L [26], while elevated TC [>5.18 mmol/L] is acknowledged as an independent risk factor for the development of coronary heart disease and cerebral vascular diseases [28]. Factors shown to affect TC levels include age, gender, heredity, HRT, sedentary lifestyle, cigarette smoking, excessive alcohol intake, overweight and obesity, diabetes, diet, and thyroid dysfunction [26,29,30,31,32]. To avoid repetition, the impact of hypoestrogenism and exercise on LDL and HDL metabolism are not discussed here, but in the relevant sections of this paper.

1.2 Postmenopausal Women and TC: Longitudinal data observing the effects of menopause on lipid parameters report increased TC, mainly due to increased LDLs and triglycerides, and decreased HDLs [39]. Such alterations in TC are predictive of mortality due to CVD [40], and are associated with aging (41) and E2 deficiency [32]. Studies have consistently shown that postmenopausal women receiving unopposed [33,34, 35] and opposed [33, 36,37,38] HRT exhibit reduced TC levels, typically through lowering LDLs, with some data also showing an

increase in HDLs, and no consistent effect observed for triglycerides. The mechanisms of E2 regulation upon LDL, HDL and triglyceride metabolism are detailed in the respective sections of this paper.

1.3 AM Athletes and TC: The majority of studies reporting TC levels in AM and EU athletes [22,42, 43,44] show no significant difference between the groups [see Table l]. However, one study, which statistically was the only study to have the power to detect differences between the groups, reported elevated TC levels in AM compared to EU athletes [20], with borderline high levels of TC seen in the AM athletes [5.49 ± 0.16 mmol/L], according to the ATP III [26] classification. Contributing factors to this elevation were not only associated with increased LDLs, as seen in postmenopausal women, but also with elevations in HDLs and triglycerides. Possible mechanisms for these individual lipoprotein elevations are discussed in the appropriate sections of this paper. The LDL:HDL ratio, identified as a strong predictor of cardiac health in men [45] was (1.7) and well below that considered a CVD risk factor [45]. The TC:HDL ratio, recently shown to be the best predictor of CVD at any TC level in women [13], was also low (2.8) for both AM and EU athletes. Again, this risk prediction calculation does not place these athletes in an "at-risk" category for CVD [13].

AM athletes consume less dietary fat intake compared to EU athletes [20, 46], although identification of types of dietary fat were not specified. This may be an important consideration when undertaking TC assessment since saturated fat has a positive and unsaturated fat a negative, correlation with serum TC [25]. Very low fat dietary intake has also been reported in anorexia nervosa (AN) patients with some reports of elevated [47] and others of normal [48] TC levels. Where increased TC is observed in AN, it has been attributed primarily to increased LDL levels [48]. The paradoxical increase in serum TC despite very low dietary fat intake has related

to an abnormality in cholesterol metabolism [49]. More specifically, it has been linked with increased synthesis of triglyceride-rich lipoproteins together with an unchanged cholesterol synthesis rate [50]. Contrary to this observation, AM athletes do not exhibit increased VLDLs (triglyceride-rich lipoproteins) when compared to EU athletes [20], and it is not known if the cholesterol synthesis rate is altered in AM athletes.

Poor overall nutritional status may have contributed to the elevated TC in AM athletes reported by Friday and colleagues [20]. This postulate can be directly related to findings in AN patients, in that those who present with the worst nutritional status also present with the greatest elevations in TC [49]. AM athletes have frequently demonstrated nutritional deficiencies [46,51, 52] compared to their EU counterparts. However, on average, the AM athletes participated in four extra hours of training per week, thereby creating a potential energy deficit. It was not reported whether the AM athletes with the greatest nutritional deficits also demonstrated the greatest alteration to cholesterol metabolism in this study. Indeed, no data currently exist to show whether AM athletes who show the greatest degree of an energy deficit also present with the least favorable TC profiles.

Strikingly similar to AN, although less marked, AM athletes also exhibit hypoglycemia and hypoinsulinemia, as well as elevated growth hormone and cortisol concentrations when compared to their EU counterpart [53]. The impact of these metabolic aberrations on TC metabolism in AM athletes is not known.

The clinical significance of altered TC levels in AM athletes is not clear. Elevated LDL with concomitantly increased HDL may preserve the cardiovascular risk profile. Whether TC concentrations are impaired due to the hypoE2ic state *per se* is not known, but it is reasonable to postulate that similar cholesterol metabolism alterations may exist between AN patients and AM

athletes due to a great number of similar metabolic and to a less extent, dietary aberrations. Further studies will help identify the extent of these possible similarities.

2.0 Low Density Lipoproteins

2.1 Introductory Comments: The LDL particle is heterogeneous in composition and variable in cholesterol content, with small, dense particles having greater atherogenicity than larger, more buoyant LDL particles [54]. Functionally, LDL plays a key role in the transport of cholesterol to all tissues, but primarily to adipose cells and the liver [23]. Approximately 60-80% of circulating LDLs are taken up by the liver via receptor-dependent mechanisms [55,56]. Apolipoprotein B, the major protein moiety of LDL and other non-HDL atherogenic lipids, acts as the ligand to the LDL receptor [54,57]. Hyperlipidemia, diabetes (type I and II), and hypothyroidism influence circulating levels of LDL cholesterol [31,58]. In addition, dietary cholesterol and fatty acids also influence circulating levels of LDLs, mediated by altering either hepatic LDL receptor activity, LDL cholesterol production rate, or both [56]. When cholesterol intake is increased, expansion of the pools of sterol, which can be synthesized *de novo*, occurs within liver cells [56]. This results in down-regulation of the LDL receptors, causing a plasma increase in the concentration of LDLs [56]. Elevated serum levels of LDLs [>3.37 mmol/L] are recognized as an independent risk factor for CVD [26,59,60], and is also associated with abnormal vasodilatory function in response to flow-mediated dilatation, facilitating the development of atherosclerosis [61].

2.2 Postmenopausal Women and LDLs: Premenopausal women have lower LDL levels compared to age-matched men [62]. After menopause, LDL levels increase, frequently surpassing those of age-matched men, with a trend toward smaller, more dense, and subsequently

more atherogenic, particle sizes [63]. The menopausal increase in cardiovascular risk is associated with unfavorable elevations in TC, triglycerides and lipoprotein (a), an LDL-like lipoprotein [12,13,54]. In an extensive review, Schwertz & Penckofer [64] identify that 25 – 50% of the potential cardioprotective effect of E2 are associated with its effect on blood lipids and lipoproteins. The impact of E2 on serum lipids derives primarily from E2-receptor-mediated effects on the hepatic expression of apolipoprotein genes [65,66,67]. Endogenous E2 [68] and E2 therapy, both transdermal and oral [69,70], reduce circulating LDL levels. This E2-associated lowering effect has been attributed to an increased catabolic rate of hepatic cholesterol into bile acids [71] and increased expression of LDL receptors on cell surfaces [72,73]. In contrast to this, the post-menopausal, hypoE2ic milieu causes reduced LDL receptor activity [54], contributing to the well documented elevation in plasma LDL concentrations in this population.

2.3 AM Athletes and LDLs: Studies observing LDL levels in AM athletes [20,42,44] are equivocal, due, in part, to variable methodologies and small sample sizes. These data are shown in Table ll. Investigators have reported significantly elevated LDL levels [20,46], as well as non-significantly different LDL levels [42,43] in association with comparable apolipoprotein B levels [43,44], in AM compared to EU athletes. It is not clear whether these alterations are of clinical significance. Interestingly, elevated LDL levels have been observed in those studies of AM athletes that show statistical significance [46] or a strong trend [20] for both reduced caloric intake and dietary fat intake.

The paradoxical increase in circulating LDL despite reduced dietary fat intake is consistent with findings in chronically hypoE2ic AN patients [49]. Normal or elevated levels of TC in AN has predominantly consisted of increased LDL levels, despite typically very low dietary cholesterol intake and a normal cholesterol synthesis rate [49]. Mechanisms for this

phenomenon in this patient group are associated with the known down regulatory effect of altered thyroid hormones; i.e. reduced total triiodothyronine (T_3), and lowered E2 levels [49] on the cellular number of hepatic LDL receptors [72,74], thereby contributing to increased plasma LDL levels [74]. Similarly, but less marked, hypoE2ic AM athletes have also displayed low total T_3 status [75,76], as well as lower caloric intake [46], and perhaps most notably, significantly less calories derived from dietary fat [20,46,77] when compared to their EU counterparts. These findings lend credence to the possibility that, although less severe than observed in AN patients, the metabolic aberrations observed in AM athletes may, in part, explain the reported elevations in LDL levels in AM athletes. This avenue of potential application merits further exploration.

It is possible that along a continuum of dietary restriction, the AM athletes that demonstrate the greatest nutritional aberrations will also demonstrate the least favorable LDL profiles. Further, down regulation of LDL receptors due to hypoestrogenism and altered thyroid status may likely play an important role in cholesterol metabolism in AM athletes, although this issue has not yet been closely examined. In addition, hypoestrogenism may prove to have a more deleterious effect upon LDL metabolism in AM athletes as a function of time i.e., the longer the episode of amenorrhea the greater the risk of elevated LDL. This possible long-term effect of hypoestrogenism has yet to be confirmed.

3.0 High-Density Lipoproteins

3.1 Introductory Comments: HDL particles correlate inversely with the risk of CVD [54], and is acknowledged as being anti-atherogenic [78]. The cardioprotection afforded by HDL in the prevention of CVD originates from the role of reverse cholesterol transport whereby HDL is

postulated to scavenge surplus cholesterol from peripheral tissues for delivery to and disposal by the liver for excretion via the bile [63,79]. Important to cellular cholesterol homeostasis, reverse cholesterol transport is proposed to be a result of apolipoprotein A-I promotion of cholesterol efflux from the cells via receptor (scavenger receptor B type I), and non-receptor (passive diffusion) mediated mechanisms [80]. Apolipoproteins A-I and A-II are the two major proteins associated with HDLs [79].

In addition to the above recognized properties, HDLs have also demonstrated anti-thrombotic [81], and favorable vascular tone [82] effects, including enhanced endothelial function [61]. The antioxidant properties of HDL can be directly related to studies that show HDL attenuates LDL oxidation [83], inhibits the atherogenic effect of oxidized LDLs [84], as well as increasing the half-life of endothelial nitric oxide [82], all of which are recognized processes that contribute to healthy endothelial function.

Taken together, these data endorse the postulation that HDLs may in some way protect against the development of atherosclerosis and heart disease via both cholesterol-dependent and –independent mechanisms.

3.2 Postmenopausal Women and HDLs: Although consistently higher in women than men during all life stages after puberty [85], HDL levels tend to decrease in postmenopausal compared to premenopausal women [86], with significant reductions effected as a consequence of menopause [39]. Bilateral oophorectomy also results in decreased circulating levels of HDLs [87]. Diminished levels of circulating E2 plays a key role in these observations, in part, due to the known E2ic stimulatory effect on apolipoprotein-A1 [88]. Evidence supporting a beneficial role of E2 upon HDL metabolism can be also be supported from examination of the exogeneous administration of E2 [89, 90]. Following E2 replacement therapy, Pickar and colleagues [89]

observed that HDL-2, the main subfraction of HDL found to increase in response to E2 therapy, resulted in enriched HDL phospholipids, thereby promoting an elevation in apolipoprotein AI production while maintaining similar metabolic clearance of HDLs. Thus, E2s not only modify HDL levels, but also its lipid composition and distribution, thereby augmenting the plasma capacity to execute cholesterol efflux [91].

3.3 AM Athletes and HDLs: Several investigators have evaluated serum HDL levels in AM athletes (Table lll). Finding have been variable, including significantly elevated [20], similar [22,42,43,46] and non-significant trends toward lower [22,42] HDL concentrations in AM athletes when compared to EU athletes. However, consistent with the observation that endurance trained athletes possess much higher HDL levels compared to sedentary populations [96], EU and AM athletes demonstrate significantly increased HDL levels compared to EU sedentary controls [46,43]. In addition, AM athletes, despite a hypoE2ic milieu, exhibit elevated HDL levels [20] such that, it can be considered a negative risk factor, thereby negating the presence of another single risk factor [26]. Friday and coworkers [20] postulate that the extra 4 hours of weekly exercise participation in the AM compared to EU athletes in their study likely contributed to these greatly elevated levels of HDLs.

Elevations of HDL levels in AM runners have also been associated with volume of exercise training, with the highest levels observed in those running the greatest distances (>64km/week). The observed improvements in HDL concentrations with increased running distance demonstrates the favorable effect of endurance exercise on cardiovascular risk, irrespective of menstrual status, and are suggestive of E2-independent mechanisms effecting the HDL increase. Indeed, the primary mechanism for the exercise-induced elevation has been postulated to be due to increased skeletal muscle and/or adipose lipoprotein lipase activity,

resulting in accelerated breakdown of triglyceride rich lipoproteins, facilitating plasma clearance and provision of free fatty acids as a fuel source for muscle metabolism or adipocyte storage [98]. However, the activity of lipoprotein lipase was not assessed by Friday and coworkers [20] and can only be speculated as a consequence of a greater volume of endurance activity in the AM versus EU athletes. Despite this, the aforementioned observations demonstrate a robust relationship between endurance exercise and HDL cholesterol concentrations, independent of menstrual status.

Women with AN have also been reported to have reduced circulating HDL levels, a consequence hypothesized to be a product of their previously discussed hypometabolic profile [93]. This observation can be explained in part by the finding that total T_3 is a potent mediator of apolipoprotein-I gene expression [94]. Consequently, when T_3 is reduced, apolipoprotein-I gene expression is also reduced, causing diminished HDL levels. Although not evaluated simultaneously in any of the studies measuring HDL levels in AM athletes, Loucks et al. [76] and others [75,95] have documented significant reductions in T_3 levels in AM compared to EU athletes. It is reasonable to suggest that the presence of reduced T_3 in AM athletes may serve as one pathway by which circulating apolipoprotein-I levels can be negatively impacted. However, apolipoprotein A-I levels have been shown to be comparable in AM compared to EU athletes [20,44], and when comparing AM athletes to EU sedentary controls [44]. It would be interesting to evaluate all of these parameters in a single study.

4.0 Triglycerides

4.1 Introductory Comments: Triglycerides (TG) are esterified fatty oils that form the core of chylomicrons and VLDL cholesterol and are comprised of a glycerol and three free fatty acid molecules [23]. TG metabolism is met by two pathways, the exogenous and endogenous cycle.

The exogenous cycle is responsible for processing dietary fat, while the endogenous cycle involves internal production of triglyceride-rich lipoprotein particles that are manufactured in the liver [99]. Recent recommendations for TC from the National Cholesterol Education Program [26] propose that TG are an independent risk factor for CVD, with desirable levels being <1.7 mmol/L. The association between elevated TG levels and CVD is not well defined and remains somewhat controversial. This is, in part, due to the close inverse metabolic association between TG and HDL [100], and the documented co-existence of elevated TG with other CVD risk factors, such as hypertension and abdominal obesity [100]. These associations have made isolated assessment of elevated TG as a predictor of CVD problematic. Further, TG measurement may not accurately reflect CVD risk status due to the lack of informative regard to the specificity of the TG-rich lipoproteins that are present in plasma [99,101]. For example, some TG-rich lipoproteins are highly heterogeneous in terms of size and lipid composition, such as, chylomicrons and large VLDL, which are thought to be unable to enter the arterial wall, and therefore considered non-atherogenic[101]. Conversely, small very low- and intermediate-LDLs can enter into the arterial intima, and as such, are considered highly atherogenic [99,101]. As such, VLDL is the most readily obtainable measure of atherogenic remnant lipoproteins [26].

A number of factors contribute to higher than normal TG levels [>1.7 mmol/L] in the general population, including obesity, being overweight, physical inactivity, cigarette smoking, excess alcohol intake, high-carbohydrate diets [>60% of energy intake], several diseases, such as type II diabetes, chronic renal failure, and nephrotic syndrome, and certain drugs including corticosteroids, E2s, retinoids, higher doses of beta-adrenergic blocking agents, as well as heredity factors [26].

4.2 Triglycerides and Postmenopausal Women: TG profiles undergo unfavorable changes in menopause and are reported to be elevated in postmenopausal women [12]. This increase can be linked to a strong relationship between excess visceral fat accumulation (android adiposity) and TG levels in postmenopausal women [102]. This type of fat distribution among women, postulated to be due to hypoestrogenism [103], is associated with unfavorable alterations in the lipid profile, such as increased LDL and TC and decreased HDL concentrations [102]. Such findings support the postulate that the co-existence of other CVD risk factors is associated with elevated TG.

At the level of the liver, orally administered HRT increases TG levels [104,105]. This is augmented by the hepatic synthesis of VLDL, particularly of the large TG-rich particles, as well as the inhibition of hepatic triglyceride lipase [15,106]. Despite this HRT-associated elevation in TG levels, concomitant favorable effects of HRT administration include decreased LDL and TC, as well as elevated HDL levels [106]. It is generally accepted that the anti-atherogenic effect of HRT upon LDL, HDL and TC outweigh the deleterious effect of elevated TG levels.

4.3 Triglycerides and AM Athletes: Studies measuring TG levels in AM athletes report non-significant [22,42,44] and significant [20] elevations, compared to the EA. Table IV illustrates these studies. Despite the majority of these findings being equivocal, observation of consistently greater TG concentrations in the AM compared to EU athletes represents what seems to be part of a 'trend' in the lipid profiles of these athletes. To date, no studies have reported assessments of TG-rich lipoproteins, such as VLDL, for the determination of atherogenic versus non-atherogenic TG particles.

Since TG are frequently associated with other CVD risk factors rather, such as low HDL, elevated LDL and TC, it is interesting to note that the one study to report significantly elevated

TG in AM athletes [20] also reported significantly elevated LDL and TC. However, the reported level of TG for AM athletes by Friday and colleagues [20] does not represent a value outside of the normal range [>1.7 mmol/L] as stipulated by the NCEP [26].

That TG was elevated in AM athletes compared to their sedentary counterparts [43,44] is consistent with data from AN patients [111] but not consistent with studies that show TG concentrations that are frequently lower in endurance athletes when compared to sedentary controls [107]. Cross-sectional data also identify similar TG concentrations in female athletes, irrespective of menstrual status and reported distance (0-139 km) run each week, [97]. These findings are suggestive of E2- and exercise-independent mechanisms in TG metabolism, although it is likely that an interaction exists between these two variables since research shows that menopause-associated hypoestrogenism increases [12] while endurance exercise decreases [107] TG concentrations. These data further highlight the potentially impaired utility of assessing TG in populations that are less likely to present with elevated TG in as much that other TG-associated CVD risk factors are absent. However, more subtle alterations to TG metabolism, such as a shift from non-atherogenic to atherogenic TG lipoprotein metabolism may be occurring, potentially necessitating closer examination of the TG composition rather than the absolute presence of TG presence *per se*.

As previously mentioned, low T_3 is associated with a pro-atherogenic lipid profile [31], suggesting that nutritional status may be an important consideration when assessing TG levels in AM athletes. A high-carbohydrate diet has been shown to increase fasting TG levels [108], while very low-carbohydrate intake has recently been demonstrated to significantly reduce fasting TG levels as well as favorably impact HDL and the TC/HDL ratios in normal weight

normolipidemic women [109]. Future research efforts should pursue the interactions among these variables.

The physiological significance of the reported elevated concentrations of TG in AM athletes is not clear. The possible mechanism behind this observation is not known. The inclusion of specific assessment of TG-rich lipoproteins may yield more pertinent information with regard to atherogenic compared to non-atherogenic TG particles.

5.0. Lipid Peroxidation

5.1 Introductory Comments: Elevated free radical production can negatively influence the oxidative status of circulating LDL particles [112]. Oxidative modification of LDL vastly elevates its atherogenicity [113] and has been implicated in the initiation and progression of atherosclerosis [114]. Increasing evidence supports that oxygen-derived free radicals, namely reactive oxygen species, are associated with destructive biological processes, including DNA and cellular membrane damage [115]. Chronic age-related disease states such as diabetes and carcinogenesis, as well as strenuous physical exercise have been implicated [116,117,118]. Reactive oxygen species incorporates hydrogen peroxide, and the less stable, superoxide- and hydroxyl- free radicals [22,119]. The magnitude of the oxidative stress is determined by the capacity of the antioxidant defenses to detoxify reactive oxygen species [120A]. Intracellular enzymatic antioxidant defenses include glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase [115,121], all of which reduce the susceptibility of the cell to potentially harmful free radicals [121]. In addition, non-enzymatic extracellular antioxidant defenses also exist, including vitamin E (alpha-tocopherol), vitamin A (beta carotene), and vitamin C (ascorbic

acid) [122]. It is the balance between reactive oxygen species production and antioxidant defenses that determines the degree of oxidative stress [119].

5.2 Lipid Peroxidation in Postmenopausal Women and Other Low E2 Models: Research surrounding lipid peroxidation and the effect of exogenous and endogenous E2 demonstrate inconsistencies. Antioxidant effects of E2 on LDL *in vivo* [123,124] and *in vitro* [114,125,126,127] have been confirmed. Conversely, no effect [128] for E2 on antioxidants *in vivo* has been documented. Differences in model utilization, subject demographics, and E2 administration, dosage, time frame, and type, have, in part, contributed to the discrepancies found in the research.

E2s may protect from atherosclerosis by inhibiting LDL oxidation [129]. Endogenous E2s have free radical-scavenging abilities, with up to 2.5 times the activity of vitamin C and E [130]. E2s ability to form moderately stable radicals from less stable radicals by donating a hydrogen atom is consistent with antioxidant function [130]. This antioxidant capability has been evidenced in premenopausal women who have significantly higher estradiol levels and lower lipid peroxide concentrations, as well as significantly higher glutathione peroxidase activity when compared to postmenopausal women [131]. In addition, significant increases in endometrial glutathione peroxidase have been observed during the high E2 phase of the cycle [132]. Such data support a beneficial effect for endogenous E2 on both intracellular antioxidant enzyme activity and free-radical scavenging abilities against lipid peroxidation.

5.3 Lipid Peroxidation in AM Athletes: There is no single biomarker that is considered the "gold standard" of lipid or protein oxidation [133]. However, evidence for oxidative stress during, and post, exercise can be obtained from measurement of free radicals, the assessment of damage to lipids, and from measurement of antioxidant redox status, particularly glutathione [134].

Considering the reported antioxidant effect of endogenous E2 [123], and exercise training [135] surprisingly few studies [22,42,136] have investigated the effects of amenorrhea on lipid peroxidation and oxidative status in AM athletes. These studies report significantly decreased LDL diene conjugation, i.e, a decreased ability of LDL to resist peroxidation [22], and a greater [42] as well as similar [136] magnitude of change for lipid peroxidation potential post-exercise in AM compared to EU athletes. Kanaley and Ji [136] also report that at rest and post-exercise, AM athletes demonstrate significantly elevated glutathine peroxidase compared to EU athletes, and that malondialdehyde, an indirect indicator of lipid peroxidation, is similar in both groups. In addition, oxysterol formation, derived from the enzymatic and non-enzymatic oxidation of cholesterol [137], is increased post-exercise in AM athletes only [22]. Collectively, these findings are conflicting and do not provide answers as to whether exercise in the face of hypoestrogenism affects antioxidant status. This can be attributed, in part, to the fact that each study utilized a different exercise protocol, including a maximal oxygen uptake test [42], as well as a 30 minute [22] and a 90 minute submaximal running bout [136]. It is also possible that the exercise intensity and/or duration of exercise may have been insufficient to stimulate an oxidant response due to the high level of aerobic fitness of the athletes. Training status of the athletes was also not consistently reported.

An important factor to consider when assessing antioxidant defense mechanisms are the training, as well as the adaptive, status of the athlete [138]. In the studies reporting the antioxidant status of AM athletes, training status was reported as mileage per week [22,136], duration of training [22], or not reported [42]. None of these studies reported the adaptive status of the athletes, thereby further compounding the lack of clarity regarding interpretation of E2 status on antioxidant status. However, despite conflicting findings, the elevated glutathione

peroxidase levels in AM compared to EU athletes [136] is indicative of an enhanced antioxidative status in the AM athlete, despite a hypoE2ic environment, suggesting alternative antioxidant mechanisms [136], such as training status. Indeed, research has shown that well trained and well adapted athletes demonstrate an augmented antioxidant system and a reduction in lipid peroxidation [135], as well as greater resistance to exercise-induced or -imposed oxidative stress [139,140,141]. Consistent with this finding is the observation that total antioxidant status is positively correlated with VO2 peak in runners [142], supporting the theory of a stress-tolerance mechanism, whereby enzymatic antioxidant defenses are enhanced due to exercise training *per se*.

In contrast to the adaptive oxidative status of the well trained athletes, higher performance and training levels are associated with greater elevations in exercise-induced lipid peroxidation [143], resulting in an antioxidant system that can become overwhelmed and unable to cope with the increased free-radical production [138]. This occurrence can be linked with overtraining [144], exercise training intensity [145], and/or the nutritional status of the athlete [146], whereby susceptibility to antioxidant deficiency may occur [146]. However, since no studies to date have confirmed an effect of E2 upon anti-oxidant status in humans in response to exercise [133], it is likely that the anti-oxidant status in AM athletes is reflective of the training and/or adaptive status, the nutritional status, and or the hypoE2ic milieu.

Consistent with previous research reporting the effect of an acute bout of strenuous exercise on anti-oxidant defenses [147], a decreased capacity to detoxify ROS after a maximal bout of exercise, as demonstrated by unfavorably altered oxysterol formation and increased lipid peroxidation levels in AM athletes [22] has been observed. These findings do imply a compromised antioxidant status in AM athletes, and that an increased potential risk for

premature atherosclerosis may be present. However, (1) a single maximal bout of exercise is not necessarily reflective of a regular training session for the athletes, and thereby not representative of the characteristic oxidative stress these athletes might otherwise incur; and (2) it is normal for oxidative stress to be elevated after strenuous exercise [147], and as such, recovery data rather than immediate post exercise data might provide more relevant information about antioxidant status. In addition, since non-enzymatic antioxidants are negatively correlated with lag time of diene conjugates [22], assessment of dietary supplementation may also be an important aspect of oxidative stress determination, particularly in the AM athlete [22].

The impact of sustained hypoestrogenism in the AM athlete upon the oxidative system is not clear, but data indicate that the antioxidant defenses of the AM athlete may be compromised after strenuous aerobic exercise. Inclusion of training, adaptive, and dietary status will help minimize confounding factors. Data suggest that in well trained individuals exercise training elicits favorable effects upon the oxidative milieu [140]. Since higher exercise intensity appears to effect a greater oxidative response in AM compared to EU athletes after maximal exercise [22], studies to determine the oxidative stress response to high exercise intensity training in AM athletes are worthy of further investigation. Mechanism(s) underlying the combined E2- and exercise-mediated protection of lipid peroxidation need to be determined. In addition, methodological consistency in measurement of antioxidant enzymes needs to be established.

6.0 Nitric Oxide

6.1 Introductory Comments: The endothelium is a multifunctional interface between the circulating blood and various tissues and organs of the body [148], and is recognized as a metabolically active organ that is vital to vascular homeostasis [149,150]. Through the

combined release of vasoactive substances, such as nitric oxide and endothelin, endothelial hemostatic function is realized. Nitric oxide is a potent vasodilator [151] that also maintains a low resting arterial tone in the peripheral [152] and pulmonary [153] circulations. Nitric oxide also inhibits platelet aggregation, suppresses smooth muscle cell proliferation and acts as an antiatherogenic factor [154,155]. Endothelial-derived nitric oxide is produced by the endothelial isoform of nitric oxide synthase upon the conversion of the substrate L-arganine to L-citrulline [155]. The production of this free radical messenger has been identified as effecting a protective role on the endothelium [150]. Impaired release and/or bioavailability of nitric oxide has been linked with hypertension [156], hypercholesterolemia [157], diabetes [158], tobacco use [159], established coronary artery disease [160], and E2 deficiency [161]. Consequently, factors that decrease nitric oxide production and/or bioavailability may promote atheroslcerosis [162].

In endothelial cells, gene expression of nitric oxide synthase (NOS), despite being constitutively activated, can also be up-regulated by both receptor-mediated (ie acetylcholine, serotonin, thrombin, bradykinin) and receptor-independent (ie shear stress) mechanisms [161]. A number of pathways leading to the release of vascular nitric oxide have been identified, including: (1) basal endothelial release that maintains low vascular tone; (2) mechanical stimuli, that is, increased shear stress; (3) dilating factors, (i.e. prostaglandins) and metabolites (i.e. adenosine) released from contracting skeletal muscle; (4) nitroxidergic and (5) cholinergic nerve stimulation, and (6) nitric oxide release from skeletal muscle [163]. The multiplicity of pathways to attain vascular nitric oxide release identifies this free radical messenger as a key modulator of vessel function.

6.2 Nitric Oxide in Postmenopausal Women

A plethora of data regarding the effect of E2 and HRT upon endothelial function in women have been published [for review, see 164]. Most [14,165,166] but not all [167] reports support a role for E2 increasing endothelium-dependent flow-mediated vasodilation. *In vitro* data also show an endothelium-independent, that is, a direct smooth muscle-relaxing effect, of 17-beta estradiol upon the coronary arteries in human females [168]. Blunted circulating levels of nitrite/nitrate [169], in addition to increased plasma levels of endothelin-1 [170], and impaired endothelium-dependent [171] and –independent [172] function occurs in postmenopausal women not receiving E2- or HRT. Further, recent data demonstrate that acute (7 days) E2 deficiency due to ovariectomy is associated with unaffected endothelial function [173], as well as impaired endothelium-dependent, but not –independent vasodilation [162] in response to flow-mediated dilation. The reason for differing findings between these studies [162,173] is not clear. Taken together these data suggest that reduced circulating levels of E2 may effect impaired endothelial-dependent and -independent function, and is associated, in part, with reduced nitric oxide production and/or bioavailability [169]. Further, 17-beta estradiol may have an important regulatory role in coronary arterial tone [168] due to possible direct effects upon endothelium and smooth muscle cells.

6.3 Nitric Oxide and AM Athletes: Despite the well documented E2-nitric oxide association, only one study documenting nitric oxide levels in AM athletes has been reported. Stacey et al., [21] observed significantly decreased levels of plasma nitrate/nitrite production despite significantly elevated dietary ingestion of nitrates in AM athletes compared to sedentary EU women. Since chronic aerobic exercise enhances blood flow and shear stress [174], and strenuous exercise can incur striking increases in plasma [175] and urinary [176] nitric oxide metabolite concentrations, it is interesting that despite participating in chronic endurance

activities, the AM athletes did not demonstrate increased nitric oxide concentrations. Consequently, the reduced plasma nitrite/nitrate levels are hypothesized to be related to the chronically low E2 status of the AM athletes [21]. The clinical consequence or physiologic relevance of this observation is not known.

Although no other studies report nitric oxide levels in AM athletes, some data alluding to the E2-nitric oxide association through observation of vascular response to flow-mediation dilation, an endothelium-dependent, and therefore a predominantly nitric oxide mediated event, does exist. Zeni-Hoch and co-workers [11] were the first to demonstrate that, similar to postmenopausal women, young AM athletes (21.9 ± 1.2 yrs) also present with endothelium-dependent, but not –independent, dysfunction compared to their oligomenorrheic and EU counterparts. Resting heart rate, mean arterial blood pressure and baseline brachial arterial diameter, were similar between the groups, yet mean percent change from baseline brachial artery diameter in response to flow mediated dilation in AM and EU athletes was 1.08 ± 0.91% and 6.38 ± 1.38 %, respectively [11]. Alarmingly, the magnitude of impaired brachial endothelium-dependent vasodilation in AM athletes is comparable to data previously reported in otherwise healthy postmenopausal women [177] and older [60 ± 2yr] coronary artery diseased patients [178] after a similar flow-mediated stimulus. Since endothelial dysfunction is a predictor of future coronary events [179], the finding from Zeni-Hoch and colleagues [11], is suggestive of increased risk for accelerated cardiovascular disease development in AM compared to EU athletes [11].

Secondary to chronically diminished circulatory E2 levels, reduced circulatory nitric oxide [ref] is a likely contributing factor responsible for the observed endothelial dysfunction in AM athletes. However, Zeni and colleagues [11] did not specifically attribute the impaired

endothelial dilatory response in AM athletes to E2 deficiency, perhaps because each group presented with similar E2 levels at the time of testing. However, chronically suppressed versus cyclical fluctuations of E2 levels would likely be better identified with daily urinary analysis of metabolites of E2 rather than a single blood draw. In addition, a single blood draw assessment cannot identify the possibility of an effect of E2 exposure across time upon endothelium-dependent function in active women with less severe menstrual disturbances, such as, anovulation, or oligomenorrhea. That oligomenorrheic and EU athletes showed similar responses for flow-mediated dilation [11] is suggestive of a "critical threshold of E2 exposure", and that some E2, as oligomenorrheic athletes are likely exposed to, is better than none, i.e., the chronically low levels AM athletes are exposed to.

An additional demonstration of the importance of E2 to vascular function can be realized from data on resumption of menses in these same AM athletes, as reported by Hoch [180]. Resumption of menses restored endothelial-dependent function in the AM athletes to levels observed for EU athletes [180]. This reversibility in endothelial-dependent function with return of ovarian function is consistent with data that show improved endothelial function in post-menopausal women after HRT [14]. Mechanisms of non-genomic vasodilatory effects of E2 includes stimulation of the opening of calcium-activated potassium channels [181], and activation of endothelial nitric oxide synthase, the precursory enzyme of endothelium-derived nitric oxide [161], via E2 receptor-alpha mediated activation [182], with heat shock protein 90 acknowledged as a key requirement to this activation [183]. Dependent pathways include E2-receptor mediated mitogen-activated protein kinase [182] and phosphatidylinositol 3 (-Akt [183]. The impact of hypoestrogenism on these pathways, however, is not well understood, but it is

reasonable to expect that AM athletes may demonstrate impairment in one or both of these pathways.

There is a positive correlation between cardiovascular fitness and endothelial function [184]. Accordingly, evidence suggests that endothelial dysfunction limits exercise capacity, either via central or peripheral mechanisms [185]. Conversely, exercise is efficacious in restoring dysfunction of the vascular endothelial nitric oxide system [186]. The exact mechanisms for this effect are not yet understood. Decreased endothelial nitric oxide may also be rate-limiting to oxygen delivery and exercise performance [187]. Data show, however, a lack of correlation between cardiovascular fitness, exercise capacity and endothelial function in AM athletes, despite being demographically similar to EU counterparts, including weekly mileage, duration of training, and 5km race time [11]. These findings imply that exercise-associated amenorrhea does not impact athletic performance, but may be implicated as a potential negator of the known cardioprotective benefits of aerobic exercise [11]. Impaired endothelial function despite chronic endurance training in AM athletes suggests that hypoestrogenism may exert a greater negative effect than the positive effect of aerobic exercise *per se*.

Mechanisms explaining the difference in endothelial function of EU and AM are not yet known, but are likely related to hormonal regulation of endothelial function which may be the result of receptor-dependent or -independent mechanisms [188]. Progesterone-, androgen-, and E2-receptors have been identified in human vascular endothelium [188]. Two functionally different receptor sub-types for E2ic actions exist, E2 receptor-alpha and -beta, although the importance of these sub-types in the vasculature remains unclear [189]. Expression of E2 receptor-alpha has been observed in human endothelium [190] and vascular smooth muscle cells [191]. E2 receptor-beta expression in vascular tissue is less well distinguished, but has been

detected predominantly in vascular smooth muscle cells [189], identifying a potential endothelium-independent role for E2. Several target genes for E2s have also been identified, including those encoding proteins that modulate lipid clearance, cardiac contractility, cell proliferation, and specifically, vascular tone [192]. Consequently, the presence of vascular E2 receptors is associated with protection against coronary atherosclerosis [193], and their expression is directly impacted by the level of circulating E2s [193].

The effect of sustained impaired endothelial function upon atherosclerotic development or vessel integrity in AM athletes awaits investigation. To date, the clinical and health implications of prolonged E2 deficiency upon the vasculature in AM athletes is not clear. Further, the time-course of decline in endothelial function, a potential "critical threshold of E2 exposure", and changes in endothelial function with resumption of menses, or perhaps exogeneous hormones, should be explored in future research.

7.0 Endothelin

7.1 Introductory Comments: Endothelial integrity, essential for normal functioning of blood vessels, is preserved not only by endogenous vasodilative, but also by vasoconstrictive substances, such as endothelins (for review, see [194]). Alteration of the nitric oxide and endothelin-1 (ET-1) systems is augmented by, and is associated with, many cardiovascular diseases [195]. The vasoconstrictive production of endothelin however, can be inhibited by nitric oxide [195]. Endothelins (ET-1, ET-2, ET-3) are biologically active peptides that oppose the effects of nitric oxide through vasconstrictive and mitogenic action [196,197]. Of the three endothelin isoforms, however, only ET-1 is constitutively produced by the endothelial cells, making ET-1 a key vascular regulator [194]. Via endothelin smooth muscle receptors,

endothelin-A (ET-A) and -B (ET-B), and endothelial cell receptor, ET-B, endothelium-derived ET-1 predominantly facilitates vasoconstriction [195], although stimulation of endothelial cell ET-B receptors can also oppose ET-A and ET-B mediated vasoconstriction by stimulating nitric oxide formation [195]. This vasodilatory response is due to increased intracellular calcium, resulting in upregulation of endothelial nitric oxide synthase [194]. In addition to vasoconstrictive and vasodilatory properties, endothelins also increase monocyte adhesion, macrophage activation, and vascular smooth muscle cell proliferation and migration through ET-A and ET-B [198]. Due to the observation that altered expression and/or activity of ET-1 can lead to the development of vascular diseases [194], ET-1 has been implicated in the progression of atherosclerosis [154].

7.2 Endothelin and Postmenopausal Women: Research surrounding ET-1 and postmenopausal women is less plentiful than that found for nitric oxide. Typically ET-1 levels have been shown to be elevated in postmenopausal women [199,200], and are significantly reduced with administration of HRT [199,200]. Chronic (6 months) E2 therapy results in decreased levels of endothelin, as well as an increased ratio of nitric oxide to ET-1 [196]. In addition, ET-1 mediated arterial constriction has been shown to be reduced after one month of treatment with estradiol [2mg/day] in older postmenopausal patients when compared to placebo [201]. This reduction is suggestive of an estradiol effect on endothelium-dependent vasoconstrictive responses, which may incorporate nitric oxide and/or prostaglandins [201]. Interestingly, long term [3 months] oral estradiol administration [2mg/day] resulted in a loss of the E2-inhibitory effect upon ET-1 mediated arterial constriction, implying possible tachyphylaxis, that is, an acute loss of response, due to sustained high doses of estradiol [201].

One potential explanation by which E2 may impart favorable anti-vasoconstrictive properties can be related to data that report estradiol inhibition of ET-1 synthesis [197], possibly through E2 receptor-dependent pathways [202]. In an in-vitro study, Dubey et al., [203] treatment of porcine coronary artery endothelial cells with varying concentrations of estradiol and estradiol metabolites, 2-hydroxyestradiol and 2 methoxyestradiol, dependently inhibited basal and serum tumor necrosis factor-alpha, angiotensin II, and thrombin induced endothelin-1 synthesis. As compared with estradiol, its metabolites were shown to be more potent in inhibiting ET-1 secretion [203]. Confirmation of such findings *in vivo* is yet to be reported.

7.3 AM Athletes and Endothelin: No studies to date have reported on the endothelin-1 levels of young (18-35yrs) AM athletes or in AN patients. However, in women of reproductive age, ET-1evels are elevated during the menses phase when compared to the follicular and luteal phases [204]. Consistent with data in postmenopausal women, endogenous E2 also appears to favorably reduce circulating ET-1. These findings lend credence to the hypothesis that ET-1 levels might be expected to be elevated in AM athletes. However, it is known that chronic exercise causes an increase in production of nitric oxide and a decrease in production of ET-1 in humans [154], which may produce beneficial effects on the cardiovascular system. Further, a significant negative correlation between plasma nitrite/nitrate concentration and plasma ET-1 concentrations has been demonstrated [154]. In support of this, data also show that exercise training diminishes the aortic sensitivity of ET-1 upon the vasculature [205], thereby necessitating less ET-1 to effect vasoconstriction or vasoregulation.

The effect of chronic exercise upon ET-1 concentrations in AM athletes is unknown. Since previous data demonstrate diminished levels of nitrites/nitrates despite chronic training and elevated dietary nitrate intake in AM athletes [21], it can only be speculated that, similar to

postmenopausal women, ET-1 levels may be elevated, possibly due to decreased sensitivity to ET-1 and/or decreased circulating nitric oxide levels.

8.0 Homocysteine

8.1 Introductory Comments: Homocysteine is an intermediate sulphur-containing amino acid produced in the metabolism of the essential amino acid methionine [206,207]. There are two major metabolic outcomes of homocysteine, the first being reversible trans-methylation [206]. This leads to the reformation of methionine, as during methionine deficiency, and ensures a sufficient supply of methionine for protein synthesis [206]. Secondly, irreversible trans-sulfuration which results in the eventual excretion of homocysteine as sulphate in the urine [206.207].

Homocysteine has been identified as an independent, modifiable risk factor for cardiovascular disease [208,209]. It is a recognized marker of systemic inflammation [13,210], and is an important mediator of atherosclerosis [207]. Mechanisms by which elevated homocysteine causes vascular disease include the direct toxic effects on the endothelium due to the generation of hydrogen peroxide formed in the process of homocysteine metabolism [206,207; 211]. This effect of increased free radical generation and subsequent lowered glutathione formation is suggested to be the principal cause for accelerated atherosclerosis [207]. Other factors important to the atherosclerotic process that are also affected by elevated homocysteine levels include proliferation of vascular smooth muscle cells [212], promotion of thrombosis [213], elevated collagen production in smooth muscle cells [214], stimulation of oxidization of LDL and anticoagulant inhibition [215]. Normal concentrations of fasting plasma homocysteine are somewhat varied, and range from 5-15 μmol/L [216], or $\leq$ 12μmol/L [217].

Hyperhomocysteinemia may derive from either nutritional (i.e. inadequate dietary intake of folate, vitamin B_{12} or $B_{6)}$ or genetic (i.e. homocystinuria) origins [206,207]. Additional factors contributing to elevated homocysteine are age, gender, impaired kidney and liver function, and certain medications [17,206].

8.2 Homocysteine in Postmenopausal Women: The homocysteine-lowering effect of E2 is well documented [17,18,211,218]. E2 status is a non-genetic factor that affects homocysteine metabolism [218]. Decreased levels of homocysteine have been reported in pregnant [219], premenopausal [208,218,220] and postmenopausal women who are on ERT [17,18,221] compared to age-matched men, surgically menopausal women, and postmenopausal women who are not on ERT.

8.3 Homocysteine in AM Athletes: There are no data available to date regarding homocycsteine levels in AM athletes. However, concerns regarding the homocysteine level of the AM athlete can be related to studies that show nutritional macro- and micro-nutrient intake of most female athletes to be less than might be anticipated based on their training load [222,223,224]. Intake of iron, calcium, vitamin B_{12} and zinc have also been reported to be below the recommended daily allowances among female athletes [223]. Since low folate concentrations have been identified as one of the key mediators of higher homocysteine levels [207], and reductions as great as 40% in plasma homocysteine concentrations with folate supplementation have been reported [225], the recommendation of sufficient dietary intake of folic acid may be particularly important for the AM athlete. Interestingly, adolescent AN patients exhibit significantly increased homocysteine levels [226]. Further, young premenopausal women also exhibit menstrual phase dependent homocysteine concentrations, with the lowest concentrations coincident with elevated E2 levels [227]. Due to the identification of an E2- and folate-lowering effect on homocysteine,

it is possible that the AM may demonstrate subtle, but potentially significantly sustained elevated levels of homocysteine. Since atherosclerosis is accelerated with even mild increments in homocysteine [228], this concern will be interesting to address.

9.0 C-Reactive Protein

9.1 Introductory Comments: Atherosclerosis has recently been identified as an inflammatory disease marker [229]. Characteristic of most forms of inflammation or tissue damage is the elevated serum concentration of acute-phase reactants, such as C-reactive protein (CRP) [230,231]. As a recognized surrogate marker of low-grade systemic inflammation, CRP reflects heightened levels of pro-inflammatory cytokines [232]. Prospective studies have shown that CRP is a strong independent risk factor for CVD [13,232], as well as a predictive tool of relative risk for future events such as stroke [233]. Specifically, data indicate that CRP predicts vascular events among low-risk groups of women with no readily apparent markers for disease [234], and even in women with LDL levels below 3.37 mmol/L [13].

Plasma CRP usually exists at very low concentrations, with 90% of individuals having a CRP <3.0 mg/L [235], but can be elevated several hundred-fold in response to infection [236]. It is important to note that as a non-specific acute phase response protein [237], CRP can be influenced by a number of factors, such as bacterial infection and inflammatory diseases [238], prolonged exercise, smoking, and age [237,238,239]. It is also positively associated with body mass index [236]. Despite the many influencing factors, CRP is still more precise than other markers of the acute-phase response, and is therefore considered an extremely useful marker of ongoing inflammation and/or tissue damage [237].

9.2 Postmenopausal Women and CRP: The reported effects of E2 and HRT on CRP levels have recently been reviewed [240]. Most [234,241,242] but not all [243] studies demonstrate an E2-mediated effect of CRP. Indeed, a recent clinical trial showed an 85% average increase in CRP over 3 years with HRT when compared to placebo [241]. This E2-increasing effect has been confirmed in cross sectional [242] and prospective [234] studies, and at first glance, suggests that HRT may be pro-inflammatory [242]. However, the increase in CRP due to exogenous HRT is postulated to be metabolic rather than inflammatory [230]. Observation of decreased inflammatory markers, such as IL-6 and E-selectin, in the presence of elevated CRP, supports a possible link with the hepatic first-pass effect of oral hormone therapies on CRP plasma concentrations [230]. This postulate makes sense when considering that plasma CRP is produced solely by hepatocytes [237]. That chronic transdermal HRT treatment does not elevate plasma CRP levels [243] further supports a metabolic rather than pro-inflammatory effect of oral HRT. The clinical relevance of these findings, however, are not known.

9.3 AM Athletes and CRP: Studies observing the effects of exercise on CRP in AM athletes have not been reported. Further, data observing CRP levels in female athletes are sparse. Fallon et al., [238] observed that an acute phase response, as determined by CRP, did not occur as a result of the levels of training typical of elite female athletes participating in court and field sports. These findings are consistent with data that demonstrate that strenuous endurance training is associated with an exercise-lowering effect on CRP concentrations [245,246]. The concept that training itself may attenuate the acute phase response, possibly by maintaining a 'balance' between response and anti-inflammation, has been demonstrated via long-term training studies that reveal a diminished acute phase reaction due to regular endurance exercise in men [245]. The decrease of the CRP base-line level after chronic training suggest that intensive endurance

exercise training may have a systemic anti-inflammatory effect, which has been postulated to be linked with an enhanced exercise-associated antioxidative defense mechanism [245]. In contrast to these findings, exercise has also been allied with an inflammatory reaction in the blood [247]. Cytoskeletal damage due to strenuous exercise can result in substantial tissue injury and clinical signs of transient immunosuppression [248]. That is, the anti-inflammatory response itself is also immunosuppressive, and can result in increased susceptibility to viral infections [248], a major stimulus of the CRP acute phase response [237].

Both endurance running and downhill running generate muscle damage [247], and unaccustomed eccentric-biased exercise, whether due to unfamiliarity of the exercise, or the intensity or duration of the exercise, incurs muscle and tissue trauma that subsequently activates an acute inflammatory response [249]. Additionally, increased free-radical production due to tissue injury can further heighten the inflammatory response [248]. Running, ballet, and gymnastics are sports typically associated with amenorrhea [250], and are associated with eccentric-biased activities that provide a high potential for muscle and tissue damage. If an enhanced oxidative defense mechanism does indeed confer generalized anti-inflammatory benefits [245], then it might be projected that AM athletes may demonstrate a compromised anti-inflammatory capacity due to reduced circulating E2, a powerful anti-oxidant in itself [251]. Together these factors may subsequently counter the chronic exercise-lowering effect on the acute phase response. Elevated levels of CRP post training for the AM athlete could, therefore, be apparent. On the contrary, chronically low E2 levels together with accustomed endurance activities may attenuate or even lower CRP levels. However, since the effect of hypoestrogenism in younger athletes upon CRP levels has not yet been explored, these hypothetical outcomes remain to be discerned.

In addition to measurement of CRP as a method of determining increased risk of cardiovascular disease, IL-6 has also been hypothesized as a good marker of cardiovascular health in apparently healthy individuals [252]. Indeed, synthesis of CRP in the liver is predominantly modulated by [253] and strongly correlated with [254] the cytokine interleukin-6 (IL-6), although IL-6 and CRP are independently related to several clinical cardiovascular risk factors in women [255]. IL-6 not only regulates immune system responses [256], but also increases fibrinogen, blood viscosity, platelet numbers, and activity [240]. Overtraining [256] and nutrient status [257] have been shown to impact pro-inflammatory cytokine levels. Consequences of a low calorie diet together with strenuous exercise stress may induce elevated IL-6 and cortisol levels in female athletes [258]. The nutrient status of the AM athlete frequently reveals suboptimal energy and nutrient intake which is linked with compromised immune responses [258], although data show no relation between susceptibility to infections and menstrual status in recreationally active women [259]. Since cytokine levels, particularly IL-6, increase with strenuous endurance activities [260], this finding suggests that exercise intensity and duration rather than menstrual status impacts the immune response. Interestingly, in malnourished individuals such as AN patients, there is an atypical, unexpected finding of lack of viral infections, or minimal symptoms in response to minor viral infections despite poor nutrient status [257]. The mechanisms behind this response have not been ascertained, but it is suggested to be a protective adaptation which is lost during refeeding [257]. Decreased IL-6 levels in AN patients [257] suggest that a lowered CRP status may be present. Whether hypoestrogenism *per se* in these patients imparts an effect on IL-6 or CRP is not clear.

In addition to decreased IL-6, persistently elevated levels of cortisol, which ordinarily reduces cytokine responses in healthy individuals, does not convey reduced inflammatory

responses in AN patients [257]. This response identifies that the feedback mechanism is somehow rendered ineffective, impairing the ability to establish an acute-phase response [257]. AM athletes also demonstrate elevated cortisol levels compared to their EU peers [261], but it is not known whether this has any impact on acute phase protein concentrations. Due to the similarity between AM athletes and AN patients, the presence of similar immune cell responses may be revealed.

10.0 Conclusion

10.1 Comments and Conclusions: The synthesis of findings secondary to the effects of hypoestrogenism in young female AM athletes upon cardiovascular outcomes discussed in this paper lend credence to the postulate that these women may be at an increased risk of premature CVD, extending the clinical sequelae of the Female Athlete Triad to cardiovascular concerns. Despite several inconsistencies with regard to statistical significance and cardiovascular outcomes, largely due to small sample sizes, it can not be dismissed that sustained unfavorable alterations to markers of cardiovascular health may prove to have long-term deleterious subclinical clinical effects. Unfavorable changes in LDL, lipid peroxidation potential, TC, TG, and endothelial function, despite a physically active lifestyle, suggest that AM athletes may potentially be susceptible to increased cardiovascular risk. This risk should be acknowledged as part of the already recognized sequelae of the Female Athlete Triad associated with amenorrhea in athletes. More studies, specifically longitudinal and prospective studies need to be performed to help discern the long-term effects of hypoestrogenism on cardiac and vascular function in young athletic women and to determine if the aforementioned unfavorable changes observed represent a clinically significant increase in CVD risk.

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