# MARKERS FOR SUBCLINICAL ATHEROSCLEROSIS IN WOMEN WITH POLYCYSTIC OVARY SYNDROME AND CONTROLS

by

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Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disorder among women in the United States. Women with PCOS experience acne, excessive hair, weight gain and irregular periods. Unfortunately, these women also have cardiovascular disease (CVD) risk factors including obesity, inflammation and type 2 diabetes. It is challenging to determine when and if atherosclerosis is accelerated in women with PCOS compared to controls as many studies investigate subclinical atherosclerosis in young women and are limited by small sample sizes. The purpose of this dissertation is to investigate markers for subclinical atherosclerosis in women with PCOS and non-PCOS controls.

The meta-analysis on carotid intima-media thickness (CIMT) showed that women with PCOS have greater CIMT compared to controls. The summary estimates of the difference are comparable to a seven year progression in CIMT. This analysis also revealed CIMT estimates were more constant across studies with higher quality assessments of CIMT.

The investigation of serum complement protein C3 (C3) suggested C3 may be an inflammatory risk marker for CVD in women with PCOS and controls. C3 was associated with traditional CVD risk factors in women with PCOS and c ontrols, and was associated with coronary artery calcium (CAC) after adjusting for case control status, age, and either insulin or

BMI. In the fully adjusted model with African American race, C3 was significantly associated with the presence of CAC.

The trajectory analysis of flow-mediated dilation in women with PCOS and controls identified three patterns of change in lumen diameter that were labeled as non-dilators, dilators and enhanced dilators. Baseline lumen diameter, insulin and HDLc were associated with group membership, and an interactive effect between PCOS status and total cholesterol on group membership was detected.

The findings from this dissertation clarify the mechanisms of subclinical atherosclerosis in women with PCOS and controls. This is of public health importance because many women with PCOS may not realize they are at risk for CVD. It is critical to evaluate factors that put these women at an increased risk of CVD so researchers can monitor risk factors and develop interventions to prevent atherosclerosis in this high risk population.

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# 1.0 DISSERTATION INTRODUCTION AND OBJECTIVES

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disorder among women in the United States. Women with PCOS experience acne, excessive hair, weight gain and irregular periods. Unfortunately, these women also have cardiovascular disease (CVD) risk factors including insulin resistance, abdominal obesity, inflammation and type 2 diabetes. These risk factors and metabolic disturbances may accelerate functional and structural impairments of the vascular system in women with PCOS. The aim of this dissertation is to investigate markers for subclinical atherosclerosis in women with PCOS and controls. Three markers of subclinical atherosclerosis were evaluated in the papers described below:

1. Many excellent reviews have discussed the association of PCOS with CVD risk factors and the risk of CVD, but a systematic review has yet to be conducted of the evidence of subclinical atherosclerosis measured by carotid intima-media thickness (CIMT) in women with PCOS. The aim of the first paper was to perform a systematic review and meta-analysis of the conflicting body of literature on CIMT in women with PCOS compared to non-PCOS controls. The results would help to summarize and interpret the literature on CIMT in women with PCOS and controls and identify sources of variability between studies.

2. Recent studies suggest that PCOS is a low-grade inflammatory state, which is concerning since atherosclerosis is classified as a vascular inflammatory disease. There has not been an investigation of the association between complement protein C3 (C3), a novel inflammatory marker, and subclinical cardiovascular disease in women with PCOS and controls. The goal of paper two was to determine whether circulating serum C3 levels were higher in women with PCOS compared to non-PCOS controls, and whether C3 levels were associated with traditional CVD risk factors and CAC in women with PCOS and controls.

3. Flow-mediated dilation (FMD) is a widely used measure of subclinical atherosclerosis, but has a lot of variability that makes it hard to interpret the results from the endothelial function assessment. Studies of FMD in women with PCOS compared to controls are inconsistent, and could be explained by the variability of the endothelial function test and studies having low sample sizes. The aim of paper three was to use trajectory analysis to identify patterns of change in lumen diameter, and evaluate the association between PCOS status and other covariates with group trajectories in women with PCOS and controls. This method may reduce the variability in analyzing FMD and could provide a new way to understand factors involved in the endothelial response after reactive hyperemia.

# 2.0 GENERAL INTRODUCTION

## 2.1 DEFINITION AND CAUSES OF POLYCYSTIC OVARY SYNDROME (PCOS)

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disorder affecting 6-10% of women in the United States.[1,2] The main features of PCOS include excess androgens, insulin resistance and obesity. Women with PCOS experience acne, excessive hair (hirsutism), weight gain and irregular periods (less than 8 per year). However, not all of these characteristics are present in a woman with PCOS, which makes it a difficult syndrome to diagnose.

The two main diagnostic criteria for PCOS have been specified by the National Institutes of Health (NIH)[3] and the European Society of Human Reproduction and Embryology/American Society of Reproductive Medicine (ESHRE/ASRM).[4] The NIH has the strictest criteria, defining PCOS as 1) androgen excess: clinical (hirsutism, male pattern baldness, acne) and/or biochemical (elevated levels of total or free testosterone); 2) ovarian dysfunction: oligoovulation (menstrual cycle length >35 days) or polycystic ovaries; and 3) exclusion of known disorders with similar features such as Cushing's syndrome, hyperprolactinemia, congenital adrenal hyperplasia, thyroid dysfunction, neoplastic androgen-secreting tumors or drug-induced androgen excess. The ESHRE/ASRM Rotterdam criterion defines PCOS as two of the three following features: 1) oligo/anovulation, 2) clinical/biochemical hyperandrogenism and 3) polycystic ovaries by ultrasound after excluding known disorders with similar features.

The Rotterdam criteria is controversial because it defines a broader patient population.[5] The Rotterdam adds two more PCOS phenotypes: irregular menses with polycystic ovaries and androgen excess with polycystic ovaries. Depending on how strict a definition, the prevalence of PCOS can range from 3% to 22%.[6] The heterogeneity in PCOS would likely lower the ability of studies to detect associations with PCOS in epidemiological studies.

To add to the complexity, not a single factor explains the pathophysiology of PCOS.[7] PCOS was thought to be an autosomal dominant trait because PCOS aggregates in families.[8,9] Legro *et al.* showed among 155 sisters of women with PCOS, about 22% had PCOS, 24% had hyperandrogenaemia but regular menstrual cycles and 54% were unaffected.[10] However, investigations have not shown a clear mode of inheritance suggesting PCOS is a complex trait such as CVD and type 2 diabetes.[5,9,11,12] Studies have evaluated genes involved in hormone and metabolic pathways. An excellent review of candidate genes in PCOS was done by Diamanti-Kandarakis and Piperi.[13] A new hypothesis suggests excess androgen exposure during fetal development changes gene expression that causes the features of PCOS.[7]

PCOS is likely a combination of metabolic, hormonal, genetic and environmental factors.[14] However, the main abnormality in PCOS is thought to be androgen excess or insulin resistance. Many investigators believe androgen excess in PCOS results from dysregulation of the hypothalamic-pituitary-ovarian axis (HPO axis)[15] or from a dysfunction in the ovary.[16] Balen *et al.* and others believe the source of androgen excess comes from abnormal theca cell activity in the ovary.[16] Theca cells in women with PCOS over respond to gonadotrophins and over express androgen producing factors.[17] Theca cells in women with PCOS produce levels of androgens 20 times higher than normal cycling women.[18]

On the other hand, Giallauria *et al.* and others believe insulin deregulation is the primary factor in PCOS.[19] Evidence shows most women with PCOS have some form of insulin resistance independent of weight.[2] Insulin causes the liver to decrease production of sex

hormone binding globulin (SHBG). SHBG binds to testosterone, thus low SHBG levels cause hyperandrogenism. PCOS has been linked to a genetic defect in insulin signaling that causes a lower response to insulin.[16] Dunaif *et al.* suggest the defect is in the signal transduction between insulin receptor substrate (IRS)-1 and the phosphatidylinositol 3-kinase (PI 3K) involved in glucose transport.[12]

## 2.2 TRADITIONAL CARDIOVASCULAR RISK FACTORS IN PCOS

Numerous studies have shown women with PCOS have higher BMI, abdominal adiposity, insulin, triglycerides, total cholesterol, LDLc, SBP and lower HDLc levels when compared to controls.[20,21] Moreover, several investigators have shown women with PCOS have a higher prevalence of CVD risk factors including higher levels of C-reactive protein (CRP),[22] endothelin-1 (ET-1)[22,23] and plasminogen activator inhibitor I (PAI-I).[24-26] Women with PCOS are also at a higher risk of developing type 2 diabetes than healthy counterparts.[27] This is concerning as insulin resistance plays a role in inflammation, endothelial dysfunction and CVD.[28,29]

## 2.3 SUBCLINICAL CARDIOVASCULAR DISEASE IN WOMEN WITH PCOS

Long-term follow up studies of CVD events in women with PCOS are limited, but several studies show women with PCOS have more subclinical atherosclerosis as measured by carotid artery intima-media thickness (CIMT)[24,25,30-35] and coronary artery calcium (CAC)[36-39] compared to controls. Also, studies report women with PCOS have impaired endothelial function, an

indicator of early vascular injury, measured by flow-mediated dilation (FMD).[22,40-42] This epidemiological evidence suggests women with PCOS have adverse structural and functional vasculature changes compared to normal menstruating women.

The following sections will review the current literature of studies evaluating the difference in FMD, CIMT and CAC between women with PCOS and non-PCOS controls. It is important to note that most of the studies were cross-sectional case-control studies.

### 2.3.1 Endothelial Function: Flow-Mediated Dilation (FMD)

Endothelial dysfunction indicates early vascular injury and is a functional measure of subclinical atherosclerosis.[24,43] The endothelium is important for vasodilation, for muscle cell growth, limiting adhesion of inflammatory cells and inhibiting platelet aggregation.[28,44-47] The endothelium relies on the balance of nitric oxide (NO) and reactive oxygen species (ROS) to maintain vasculature tone.[48] Epithelial cells overcome sheer stress by releasing NO to relax smooth muscle cells thereby increasing the lumen diameter.[23,49]

Endothelial function and can be measured by brachial flow-mediated dilation (FMD). FMD measures changes in brachial artery diameter in response to increased flow after transient ischemia (sheer stress).[50] A low FMD in response to an increased blood flow indicates endothelial dysfunction. The most commonly used method to measure FMD involves a sonographer that places a tourniquet on the participants forearm and uses B-mode ultrasound to measure the baseline advential diameter of the brachial artery. The sonographer inflates the tourniquet 50 mmHg above the participant's SBP for 4 minutes. The sonographer deflates the tourniquet and measures brachial advential diameter for 2 minutes after deflation. FMD is expressed as a percentage and is calculated by: [(maximum diameter after deflation-baseline diameter)/baseline diameter] x 100.

Endothelial dysfunction has been shown in animal models of atherogenesis[51] and in individuals with atherosclerosis.[50,52] Previous studies estimate FMD is around 5% to 15% in most individuals, but lower or missing in individuals with cardiovascular disease.[49] The Cardiovascular Health Study showed the mean FMD was lower among 743 participants with clinical CVD (2.93%) compared to 1441 participants without CVD (3.13%; p=0.025) after adjusting for important CVD risk factors.[53] Endothelial dysfunction is associated with age, BMI,[54] hypertension,[55,56] inflammation[29,57] and metabolic factors seen in PCOS such as dyslipidemia and insulin resistance.[43,58,59]

In addition to these CVD risk factors, potential confounders that affect endothelial function include race,[60] menstrual cycle,[61] estrogens and menopausal status,[62,63] exercise,[44] alcohol,[64] tea,[65] cocoa,[66] smoking,[67] stress,[68] and psychosocial factors including Bortner Type A behavior, Spielberger trait anger and Beck depression scores.[69] Endothelial function may vary by the time of day, but this has not been consistently shown.[48,70] Medications such as ACE inhibitors, statins, antioxidants, insulin sensitizers and L-arginine improve endothelial function.[44,71] HDLc, adiponectin, vitamin B and folic acid are also positively related to endothelial function.[26, 43,59]

Studies of FMD in women with PCOS compared to controls are inconsistent (see Appendix A). Most studies enroll premenopausal women and have sample sizes. The four largest studies, with more than 50 cases, show women with PCOS have statistically lower FMD compared to controls.[22,33,42,72] Cascella *et al.* studied 200 women with PCOS (Rotterdam) and 100 age- and BMI-matched controls with a mean age of 24 years and a mean BMI 29 kg/m$^2$.[22] The women with PCOS had a mean FMD of 13.7% versus 17.8% among controls (p<0.001). Investigators consecutively enrolled patients with PCOS through the Department of Molecular and Clinical Endocrinology and Oncology in Naples, Italy, and enrolled healthy controls. However, they did not specify who the controls were or how they were identified.

Similarly, Carmina *et al.* showed FMD was 15.0% among 50 women with PCOS (NIH) versus 18.2% among 50 age- and weight-matched controls ($p<0.05$).[33] Carmina *et al.* did not specify how the women with PCOS and controls were enrolled. The two other studies showed a lower FMD among 62 cases (Rotterdam) and 17 controls[42] and among 100 cases (NIH) and 20 controls.[73] However, these studies are limited because they only measured the post-deflation maximum diameter at one minute.

Studies showing no differences in FMD between cases and controls are limited by small sample sizes ranging from 10 to 40 cases.[74-80] Soares *et al.* enrolled 40 women with PCOS (Rotterdam) immediately after diagnosis at the University of Sao Paulo Hospital, Brazil and 50 controls from a basic health unit (mean age 24.5 years and mean BMI 23 $kg/m^2$).[80] They showed FMD was 8.1% among cases and 8.4% among controls ($p=0.80$). However, they only measured the post-deflation maximum diameter at one minute. In a similar population, Arikan *et al.* enrolled 39 women newly diagnosed with PCOS (Rotterdam) at the Endocrinology Department of Medical School of Dicle University, Turkey and 30 age- and BMI-matched controls from an outpatient clinic.[77] The mean FMD among cases was 24.9% compared to 22.4% among controls ($p>0.05$). However, these results are questionable because the mean FMDs are surprisingly high.

The Cardiovascular Health and Risk Measurement (CHARM) study is the only investigation of FMD in middle aged women, some postmenopausal, with PCOS and controls. Talbott *et al.* showed no difference in FMD between cases and controls in a subsample of 211 women from CHARM II from 1997 to 1999. The mean FMD was 7.33% in 95 women with PCOS (NIH) compared to 7.15% in 116 cases.[81] However, the women were over 30 years old, with a mean age of 43, and menopausal status or hormone use was not reported in this analysis.

The limitations of these studies of FMD and PCOS include low statistical power, the study design and methodology of FMD. The broad Rotterdam criteria would lower a studies power to see an effect of PCOS. Four of the seven studies that did not detect a difference in FMD between cases and controls used the Rotterdam criteria. In addition, most studies had inadequate power to detect a difference in FMD between cases and controls due to very low sample sizes.

Some of these case-control studies failed to select a representative control group. Studies enrolled patient or community controls,[73,76,80-82] doctors, medical students and nurses as controls.[23,40,41,77,83,84] These controls could be healthier than an average woman, thus overestimate the difference in FMD between cases and controls. Studies that recruited "healthy controls" may have potential biases such as self-selection bias and the healthy worker effect. These studies did not specify how the controls were recruited or where they came from.[22,42,75,78,79,85-87] Also, studies did not report the response rate for recruitment that could be a source of selection bias.

These studies compared the mean FMD between cases and controls and did not use regression analyses to determine if PCOS is related to FMD independent of the potential confounding of important CVD risk factors. Some investigators did not adjust for differences in BMI[82] or age.[41,75,88] Other important factors related to FMD were not addressed including hormone use[41,75-77,82,86], smoking [22,78,84,86,88] and differences in baseline lumen diameter. Some studies did not report the baseline lumen diameter.[22,41,73,75,76,79]

Since there is not a standardized protocol of FMD, it is not surprising the methodology of FMD varied across studies. However, there were some differences that limit the ability to compare results across studies. The occlusion time in these studies ranged from three to five minutes. Studies with longer occlusion time may have higher FMD because the longer duration of occlusion is associated with a higher FMD.[89] Another issue is when investigators measure

the post-deflation diameter. Some studies measured the maximum post-deflation diameter continuously, whereas others measured the diameter at 20 second or 30 second intervals, or measured one post-deflation diameter at one minute.[30,42,59,79,80] The true maximum diameter may not be measured in these studies as fewer time points are used. Alexandraki *et al.*[84] did not describe the methods and Lowenstein *et al.*[41] used different equipment (Endo-Pat).

The differences in FMD methodology would lead to misclassification. However, the misclassification would occur in cases and controls (non-differential). Thus, studies would have a harder time to detect a difference in FMD between groups.

### 2.3.2 Carotid Artery Intima-Media Thickness

The thickness of the intima-media in the common carotid artery is a structural measure of subclinical atherosclerosis. Carotid intima-media thickness (CIMT) is measured from B-mode ultrasound and is a strong predictor of CVD events [90-93] and CVD risk factors. [94,95] The Cardiovascular Health Study followed 5,858 participants for an average of 6.2 years, and found every one standard deviation increase in CIMT had a relative risk of 1.3 (95% CI 1.23-1.52) for myocardial infarction and relative risk of 1.33 (95% CI 1.20-1.47) for stroke after adjusting for CVD risk factors.[96 91]

The majority of epidemiological studies show women with PCOS have higher CIMT values than controls;[24,26,31,34,97-105] one study showed this observation only in older women.[106] However, some studies show no difference in CIMT between cases and controls.[30,77,80,84,107-111] Most studies enrolled premenopausal women with the exception of the study by Talbott *et al.*[112]

Cascella *et al.* conducted the largest study of CIMT in 200 cases (Rotterdam) and 100 age- and BMI-matched controls with a mean age of 24 years and mean BMI of 28 kg/m$^2$.[101] They showed the mean CIMT was 0.46 mm among cases and 0.38 mm among controls

($p<0.001$). In a population with a similar age and BMI, Carmina *et al.* also showed the mean CIMT among 95 cases (Androgen Excess Society criteria) was 0.61 mm compared to 0.53 mm among 90 controls ($p<0.01$).[26]

However, Erdogan *et al.* showed no difference in CIMT between 68 cases (Rotterdam) and 26 controls with a mean age 25 years and mean BMI 24 kg/m$^2$.[109] CIMT was also similar between cases and controls in a slightly older and heavier population (mean age 33 years, mean BMI 37 kg/m$^2$). The mean CIMT was 0.55 mm among 100 cases (NIH) compared to 0.54 mm among 20 controls ($p>0.05$).[30]

In the CHARM study, there was no difference in the age- and BMI-adjusted mean CIMT between 125 cases (NIH) and 142 controls (0.70 versus 0.67 mm, $p=0.30$, respectfully).[112] However when stratified by age, a significant difference in CIMT was seen between cases and controls in women over 45 years old, but not between women 30-44 years old. Among women 20-44 years old, 78 cases had an age and BMI-adjusted mean CIMT 0.65 mm compared to 0.64 mm among 82 controls ($p=0.565$). Among women 45 years and over, 47 cases had an age- and BMI-adjusted mean CIMT of 0.77 mm compared to 0.71 mm among 60 controls ($p=0.005$). In linear regression models, PCOS remained significantly associated with CIMT (beta=0.206, $p=0.042$) after adjusting for age and BMI.

Similar to the limitations of the studies investigating FMD, the different associations of CIMT and PCOS in these studies is likely due to low statistical power, the study design and methodology of CIMT. The broad Rotterdam criteria would lower a studies power to see an effect of PCOS. The sample sizes ranged from 16 to 200 so the smaller studies may have lacked sufficient power to detect a difference in CIMT between cases and controls.

A few studies enrolled patients[30,80,99,110] and community controls.[34,97,112] Some studies may have potential biases due to the control group. Studies enrolled doctors, medical students and nurses as controls,[31,77,84,98,102,103] and some did not mention where the "healthy controls"

came from or how they were recruited.[24,26,100,108,109,111,113] Also, the response rate of the recruitment was not reported in any study that could lead to a potential selection bias.

Most studies controlled for important confounders between women with PCOS compared to controls. Only a few studies did not adjust for significant differences in age and BMI between cases and controls[98,102,111] and some studies failed to assess hormone use.[77,97-100,102,112] The protocol for CIMT varied across studies; however in contrast to FMD, CIMT is more reproducible so the variability is less of a concern than with studies assessing FMD.

### 2.3.3 Coronary Artery Calcium

The presence of coronary artery disease, or coronary artery calcium (CAC), is evaluated by using electron beam computed tomography (EBCT). CAC has been shown to indicate the extent and severity of CVD above that of traditional CVD risk factors, C-reactive protein and CIMT.[114-116] Moreover, CAC predicts CVD events better than the Framingham risk score.[114,115,117]

Three studies showed the prevalence of CAC, dichotomized as none versus any, was higher in premenopausal women with PCOS compared to controls. Christian *et al.* enrolled 36 cases (NIH) and 71 age- and BMI-frequently matched community controls. The mean ages were 38.5 for cases and 39.0 for controls and the mean BMI was 31.4 for cases and 31.2 for controls. The prevalence of CAC was 39% among 36 cases compared to 21% among controls (odds ratio 2.37; p=0.05).[38] Mean CAC scores were also higher among cases versus controls (8.9 versus 1.7; p=0.03, respectfully). PCOS was not independently associated with the prevalence of CAC after adjusting for BMI (odds ratio 1.99; p=0.21), or associated with the mean CAC score (p=0.26) after adjusting for BMI, waist circumference, fasting glucose, HDLc and triglycerides.

Shroff *et al.* enrolled 24 cases (NIH) from the PCOS clinic at the University of Iowa and 24 age- and BMI-matched controls from annual exam visits at the Gynecology Clinic and by advertisements in the hospital newspaper. The prevalence of CAC was 33% among cases versus 8% among controls (odds ratio 5.5; p<0.03).[37] However, they may have a potential selection bias for excluding chronic illnesses like asthma, inflammatory bowel disease, or any illnesses within a month preceding the study.

The Cardiovascular Health and Risk Measurement Study (CHARM) is the only prospective study that was able to measure CVD risk factors nine years before the CAC assessment.[39] The prevalence of CAC was 45.9% among 61 women with PCOS (NIH) versus 30.6% among 85 controls of similar age (p=0.059). PCOS remained a statistically significant predictor of CAC after adjusting for age and BMI (odds ratio 2.31, p=0.049), but not after adjusting for triglycerides, insulin or HDLc. The women were 35 to 62 years old, but menopausal status was not assessed in this analysis.

In a cross-sectional analysis of women 35-60 years old from the 2000-2003 follow-up, the prevalence of CAC was 63.1% in 149 cases versus 41.0% in 166 controls (p=0.037) after adjusting for age and BMI.[36] PCOS status remained statistically significant in a logistic regression model of CAC (CAC ≤10 versus >10) after adjusting for age, BMI, HDLc, fasting glucose and menopausal status. In addition to PCOS, fasting glucose (OR 1.04, p=0.009) and menopausal status (natural menopause OR 3.7, p=0.008; surgical menopause OR 3.13, p=0.037) significantly predicted CAC.

## 2.4 THE CARDIOVASCULAR HEALTH AND RISK MEASUREMENT STUDY (CHARM)

Two papers in this dissertation were based on the Cardiovascular Health and Risk Measurement Study (CHARM) that has been ongoing since 1992 (Dr. Evelyn Talbott *et al.*). The aim of CHARM I was to evaluate the prevalence of CVD risk factors in women with PCOS compared to controls. Since then, there have been three phases of the CHARM study (Table 2.1).

In CHARM I, investigators identified women aged 19-55 years old diagnosed with PCOS between 1970 and 1993 through medical records in the Division of Reproductive Endocrinology at Magee-Women's Hospital (Pittsburgh, PA).[20,39] Investigators used NIH criteria to define PCOS and matched cases to neighborhood controls by age ±5 years and race using voter's registration tapes and the Cole's Cross Reference Directory of Households from 1993.[3]

From CHARM I, Talbott *et at.* showed women with PCOS had significantly higher BMI, LDLc, insulin, triglycerides, waist hip ratio and lower HDLc than age matched controls.[20] Subsequent reports showed the difference in CVD risk factors between cases and controls differed by age group.[81,118] When stratified by <45 years old versus ≥45, younger women with PCOS had higher LDLc, total cholesterol after adjusting for BMI, hormone use and insulin than controls. There were no differences between cases and controls after 45 years old. Similar results were found using age 40 as the cutoff.[118]

In CHARM II and CHARM III, Talbott *et al.* evaluated markers of subclinical atherosclerosis that included CAC, CIMT and FMD in women with PCOS and cases over 30 years old. As described in the literature review above, women with PCOS had greater CIMT[81] and CAC[39] compared to controls, but no difference in FMD.

From CHARM III, Talbott *et al.* showed 149 women with PCOS had a higher risk of developing type 2 diabetes than 166 controls.[27] Among all races aged 35-64 years, the relative

risk of type 2 diabetes associated with PCOS was 2.38 (p=0.06) after adjusting for age and BMI group. The population attributable risk percent of type 2 diabetes associated with PCOS was 7.6%, assuming the prevalence of PCOS is 6% in the general population. For Caucasian women aged 40-59, the relative risk of type 2 diabetes associated with PCOS was 3.95 (p=0.03) adjusted for age and BMI group. The population attributable risk percent of type 2 diabetes associated with PCOS was 15%, assuming the prevalence of PCOS is 6% in the general population.

Future follow-up of the women in CHARM will allow investigators to evaluate the risk of CVD events among women with PCOS and controls. Future studies will also be able to assess the role of menopause and subclinical atherosclerosis in these women.

## 2.5 TABLE FOR CHAPTER TWO

Table 2.1 Phases of the CHARM study (1992-2006)

| **Phase** | **CHARM I 1992-1995** | **CHARM II 1997-1999** | **CHARM III 2002-2006** | | |
|---|---|---|---|---|---|
| | | | Visit 1 2002-2003 | Visit 2 2002-2004 | Visit 3 2003-2006 |
| Participants | N=486<br>243 cases and 243 controls | N=335<br>161 cases and 174 controls | N=328<br>157 cases and 171 controls | N=228<br>108 cases and 120 controls | N=276<br>125 cases and 151 controls |
| Measurements | Fasting blood draw, medical history and anthropometric measures | Fasting blood draw, medical history, anthropometric measures (CT-measured) and subclinical CVD measures (CIMT, FMD) | Fasting blood draw, medical history and anthropometric measures and EBCT (CAC) | Subclinical CVD: PWV (N=212), CIMT (N=225) and FMD (N=187) | Fasting blood draw and repeat EBCT (CAC) |
| | | Repeat CIMT and FMD 5 yrs later CHARM III Visit 2 | Repeat EBCT 3 yrs later CHARM III Visit 3 | | |

*Fasting blood draw included hormone, lipid and metabolic panels as well as selected inflammatory, fibrinolytic and coagulation factors; anthropometric measures included BMI, waist-hip-ratio and waist circumference. CT: computed tomography; CIMT: carotid intima-media thickness; FMD: flow-mediated dilation; PWV: pulse wave velocity; EBCT: electron beam computed tomography

## 2.6 REFERENCES FOR CHAPTER TWO


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# 3.0 CAROTID ARTERY INTIMA-MEDIA THICKNESS IN POLYCYSTIC OVARY SYNDROME: A SYSTEMATIC REVIEW AND META-ANALYSIS

## 3.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) is a reproductive endocrine disorder that affects approximately 7 million women, about 6-10% of women, in the United States.[1,2] Women with PCOS experience acne, excessive hair, weight gain and irregular periods. Unfortunately these women also have an increase in cardiovascular disease (CVD) risk factors including insulin resistance,[3] dyslipidemia,[4] abdominal obesity,[5] type 2 diabetes[6] and inflammation.[7] These risk factors and metabolic disturbances may cause functional and structural impairments of the vascular system in women with PCOS and have long-term effects on the process of atherosclerosis as these women age.

The extent to which there is an increased risk of subclinical atherosclerosis and CVD events among women with PCOS remains controversial. Studies of CVD events in women with PCOS are limited, but a recent meta-analysis showed women with PCOS had two times the risk of coronary heart disease or stroke than controls.[32] Some studies have found that women with PCOS had greater subclinical atherosclerosis as measured by coronary calcification (CAC),[8-11] carotid artery intima-media thickness (CIMT)[12-18] and endothelial dysfunction measured by flow mediated dilation (FMD)[5,19-21] compared to controls, but not all showed a significant difference.[22-25] Most of these investigations for subclinical atherosclerosis evaluated young women of

reproductive age, a time generally early for the detection of atherosclerosis, and were limited by small sample sizes. This provides a challenge to determine when and if atherosclerosis is accelerated in women with PCOS compared to controls.

Many excellent reviews have discussed the association of PCOS with CVD risk factors and the risk of CVD,[26-31] but a systematic review has yet to be conducted of the evidence of subclinical atherosclerosis in women with PCOS. Thus, the aim of this study was to review the literature regarding CVD risk assessment by CIMT in women with PCOS compared to controls. CIMT is a non-invasive ultrasound measure of the thickness of the intima-media of the common carotid arteries. It is a widely used structural marker of subclinical atherosclerosis that is associated with CVD risk factors[33,34] and CVD events.[35-38] This report follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[39,40]

## 3.2 METHODS

### 3.2.1 Eligibility Criteria

Primary articles investigating CIMT among women with PCOS and non PCOS controls were included if they: (1) were a peer-reviewed primary article, (2) had a study population of women with PCOS (diagnostic criteria for PCOS specified by the National Institutes of Health (NIH),[41] the European Society of Human Reproduction and Embryology/American Society of Reproductive Medicine (ESHRE/ASRM),[42] and/or the Androgen Excess Society (AES) criteria),[43] and were compared to non PCOS controls, (3) reported a measure of CIMT (unadjusted or adjusted), and (4) were published in the English language. We excluded studies without a control group.

### 3.2.2 Search Strategy and Study Selection

Papers assessing CIMT in women with PCOS were identified using Ovid MEDLINE, EMBASE and PUBMED. The primary search was conducted in Ovid MEDLINE through November 19, 2010 (MLM). The search terms for Ovid MEDLINE included carotid artery diseases, tunica media, carotid artery, common/tunica intima, arteriosclerosis, intima-media thickness and polycystic ovary syndrome: physiopathology, pathology, complications, etiology, mortality, ultrasonography, epidemiology, prevention and control (Figure 3.1). The search terms for EMBASE were intima-media thickness, ovary polycystic disease limited to humans and limited to the publication years to 1980-2010. A search through PUBMED did not identify any new references. Two independent investigators reviewed reference lists from primary search (MLM, EOT). Review papers were assessed to find possible references not identified in the Medline and EMBASE journal databases. Before finalizing this meta-analysis, a search in PUBMED identified an additional study published in 2011.

### 3.2.3 Data Extraction

The data from the studies was extracted into a table and re-verified (MLM). The information from each study included: first author, journal, study design, whether the study controlled for age and BMI or weight, PCOS diagnostic criteria used, source of the control population, number of participants, mean age and BMI of the participants, measure of CIMT (both unadjusted and adjusted were extracted if reported), p-value for the difference between cases and controls, and the methodology for the CIMT measurement (carotid segments used, the calculation of CIMT and reproducibility information).

Two investigators met to discuss eligibility of studies to be included in the meta-analysis (MLM, EOT). In any cases of disagreement, a third arbitrator was consulted (RAW). Two of the three investigators were required to be in agreement. After reviewing articles, 26 studies met the inclusion criteria and 19 studies were suitable for the meta-analysis (Figure 3.2). One article published in 2011, as mentioned previously, was added to the meta-analysis.[44] For the statistical analysis, three studies were excluded that did not report necessary information for the meta-analysis,[25,45,46] one paper for reporting unusual CIMT values[47] and four papers that were from the same author or study population.[48-51] In instances of duplicate papers from the same first author, the most recent study containing the larger sample size was included in this meta-analysis.

### 3.2.4 Assessment of Risk of Bias

Two investigators independently assessed limitations and possible biases within each study (MLM, EOT). This information was used to determine if studies were adequate for the meta-analysis and to determine possible sources of heterogeneity. A priori, it was hypothesized that the studies might differ according to the protocol and reproducibility of CIMT and the PCOS diagnostic criteria that was used. Publication bias across studies was assessed using a funnel plot and Egger's test.

### 3.2.5 Data Analysis

The primary outcome of interest was the mean difference in CIMT between women with PCOS and non PCOS controls. The meta-analysis was performed using a random-effects model to compute the mean difference in CIMT and the 95% confidence intervals for each study

and an overall summary estimate. The mean CIMT, the standard deviation and the sample size were available for most of the studies. Four studies were included that reported means and exact p-values[44,52,53] and three studies were included that used an adjusted mean CIMT.[13,14,52] Three studies were ineligible to be included for the meta-analysis because they did not report the necessary information.[25,45,46] One study was excluded because the reported CIMT was unusually low in both women with PCOS and controls.[47] One study stratified cases by levels of the homeostasis model assessment-insulin resistance (HOMA-IR) but not the control group.[54] In this case, the information from the case group that was most similar to controls was used to give a conservative estimate. The right CIMT results were used for three studies that reported the left and right CIMT separately.[54-56]

Forest plots were created with the random effect model to obtain an estimate of the overall mean difference in CIMT across the studies. The random effects model was used to incorporate greater variability or heterogeneity between the studies. The a priori hypothesis was that the heterogeneity may be due to differences in PCOS diagnostic criteria, the age and BMI of the study populations, the protocol for CIMT and the observer variation of the technician(s) performing the ultrasound assessments. B eyond visual assessment for heterogeneity, homogeneity was tested using the chi-square test Cochran's Q-statistic. A p-value of <0.10 was used to suggest heterogeneity. The $I^2$ statistic was computed to measure the proportion of inconsistency that could not be explained by chance in each of the individual studies.[57] $I^2$ ranges between 0% and 100% with lower values representing less heterogeneity. The recommended guidelines for low, moderate and high $I^2$ values are <25%, 50% and >75%, respectfully.[57] However, the power to detect bias is under 0.80 with a meta-analysis of less than 20 studies and including studies with less than 80 participants.[58-60]

To examine possible sources of heterogeneity between studies, the meta-analysis was conducted by grouping the studies by the quality of the CIMT measurement. The quality of the

studies was determined by evaluating if the study reported reproducibility of CIMT and if the study used an average of the left and right common carotid artery (CCA) for CIMT versus just one side. This criteria was used because the average of measures from the left and right CCA would be more stable than the average of one side.[61] Finally, to assess possible publication bias, a funnel plot was created to assess for symmetry and the Egger regression test was performed to test for asymmetry of the funnel plot. The Egger test evaluates the association between the standardized effect estimate (estimate divided by standard error) and the precision (1/standard error) through the use of linear regression for Y-intercept=0.[62] All analyses were performed using Comprehensive Meta-Analysis software (Borenstein M, Hedges L, Higgins J, Rothstein H. Comprehensive Meta-analysis Version 2, Biostat, Englewood NJ (2005)).

## 3.3 RESULTS

### 3.3.1 Description of Studies

A total of 56 articles were identified for the review through a database search of Ovid MEDLINE and EMBASE after adjusting for duplicates (Figure 3.2). The investigator screened the title and abstracts and excluded 20 articles because they were reviews, did not have a control group or did not report a CIMT measurement. There were 36 full-text studies that met the eligibility criteria for this systematic review and meta-analysis. Of these, 10 studies were excluded because they were not in English, did not have a control group, or did not report a CIMT value. Eight studies were excluded from the meta-analysis because they had the same first author or study population, the necessary statistics were not reported or the CIMT values were abnormal compared to the other studies, as previously mentioned. In summary, the

search identified 27 studies for the systematic review and 18 studies for the meta-analysis. An additional article published in 2011 was identified and subsequently added to the meta-analysis, for a total of 19 studies to be included in the meta-analysis.

The majority of the 27 studies included in the systematic review were cross-sectional studies with the exception of one randomized clinical trial[17] (Table 3.1). The details of each study are shown; however this paper focused on studies that were included in the meta-analysis (Table 3.2). The 19 studies in the meta-analysis involved a total of 1123 women with PCOS and 923 non PCOS controls. The sample sizes ranged from 18 to 200 women with PCOS and from 12 to 124 controls. Women with PCOS were diagnosed using the NIH criteria, the AES criteria or the Rotterdam criteria. The studies enrolled patient,[25,45,54,63] community controls,[17,48,52] doctors, medical students and nurses as controls.[13,22,53,55,64,65] Some studies did not specify where the healthy controls came from or how they were recruited.[14,24,44,49-51,66,67]

The studies enrolled women with a mean age range from 22 to 40 years old and a mean BMI range from 21 to 30 kg/m$^2$. The women were premenopausal with the exception of one study.[52] All but four[44,64,65,67] studies matched or adjusted for age and BMI or weight between women with PCOS and controls for the CIMT estimate. CIMT was assessed using B-mode ultrasound of the common carotid artery and calculated as a mean of measurements of the far wall of the left and right common carotid artery. One study used the maximum CIMT.[44] Most studies averaged the right and left CCA together, whereas a few reported them separately. The mean CIMT ranged from 0.41 to 0.75 mm in women with PCOS and from 0.33 to 0.74 mm in non PCOS controls.

Quality control measures for CIMT were reported in nine studies. The most common reported reproducibility statistic was the intra-observer coefficient of variation (CV). The CV shows the variability between measures where low CV values indicate less variability in the measures. The intra-observer CV for the seven studies were <11%,[5,13,14,17,44,66,68] and the inter-

observer CV for one study was 12%.[14] One study reported an intra-class correlation coefficient (ICC) of 0.86[12] where higher values indicate more measurement variability is due to differences between patients rather than other sources of error. One study reported an intra-observer error of $< 0.03$ mm.[18] The remaining 11 studies did not mention the quality control measures for CIMT.[23,54-56,65,69] But, five of these studies indicated there was one technician reading the CIMT images.[22,24,47,53,67]

From this information, the studies were ranked by the quality of the CIMT assessment. There were eight studies considered to be of highest quality because they reported a reproducibility statistic and used the left and right CCA for CIMT.[5,12,14,17,18,44,68] Two studies were considered the next highest quality because they reported a reproducibility statistic and used one CCA for CIMT.[13,66] Five studies were considered to be of fair quality because they did not report a reproducibility statistic and used the left and right CCA for CIMT.[22,24,53,65,67] The remaining six studies were the lowest quality because they did not report a reproducibility statistic and used one CCA for CIMT.[23,54-56,69]

### 3.3.2 Risk of Bias

The funnel plot of the eight highest quality studies suggested no evidence of publication bias because the studies were symmetrical around the mean and the Egger's regression test was not significant at p=0.94 (Figure 3.3). Publication bias was not assessed among the two studies of the next highest quality as this requires more than three studies to run publication bias procedures. The funnel plots of the fair and lower quality studies were also symmetrical around the mean (data not shown) and the Egger's test p-value was 0.48 for the five studies without reproducibility and used the right and left CCA, and the p-value was 0.61 for the six studies without reproducibility and used one CCA.

### 3.3.3 Mean Difference in CIMT

The forest plots showed that the mean difference in CIMT between women with PCOS and controls varies across the groups of studies (Figures 3.4 to 3.7, Table 3.3). The forest plot of the eight studies of the highest quality suggested cases had a greater CIMT than controls as most of the estimated difference in means, except one, were to the right of zero (Figure 3.4). The widths of the 95% confidence intervals (CIs) were similar, which indicated the studies had similar precision in the estimates. The summary random effect mean difference in CIMT showed that PCOS women had a s ignificantly greater CIMT than controls (0.072, 95% CI [0.040, 0.105], p<0.0001). The Q-statistic for heterogeneity was significant ($\chi^2$=36.82, p<0.0001, $I^2$=80.99). However, as noted above, these heterogeneity estimates should be interpreted with caution because they are not reliable with a small number of studies and small numbers of participants within some studies.

Like the previous studies, the forest plot of the two studies of the next highest quality showed that the estimated difference in means and 95% CIs were similar and were located to the right of zero (Figure 3.5). The summary random effect mean difference in CIMT showed that PCOS women had a significantly greater CIMT than controls (0.084, 95% CI [0.042, 0.126], p<0.0001). The Q-statistic for heterogeneity was not significant ($\chi^2$ =0.05, p=0.82, $I^2$=0.00). This signifies similarities or homogeneity between the studies, but this is based on two studies.

The forest plot of the five studies of fair quality showed that most of the estimates and 95% CIs cross zero, except for one study (Figure 3.6). Two studies had a wide CI compared to the rest, which indicated less precision in the estimate. In contrast to the highest quality studies, the estimates of the difference in CIMT across studies did not show a consistent pattern. The summary random effect mean difference in CIMT was not significant between women with

PCOS and controls (0.041, 95% CI [-0.038, 0.120], p=0.310) and the Q-statistic for heterogeneity was significant ($\chi^2$ =30.11, p<0.0001, $I^2$=86.72).

Similar to the fair quality studies, the forest plot of the six studies of lower quality showed that most of the estimates were around zero and the 95% CIs crossed zero, except for one study (Figure 3.7). Similar to the fair quality studies, there was not a consistent pattern in the estimated difference in means and CIs across studies. The summary random effect mean difference in CIMT was not significant between women with PCOS and controls (0.045, 95% CI [-0.020, 0.111], p=0.173) and the Q-statistic for heterogeneity was significant ($\chi^2$ =43.58, p<0.0001, $I^2$=88.53).

## 3.4 DISCUSSION

### 3.4.1 Summary of Evidence

Overall, this meta-analysis suggested that women with PCOS have a higher mean CIMT compared to non PCOS controls. The summary estimate of the mean difference in CIMT was 0.072 mm for women with PCOS compared to controls (95% CI [0.040-0.105], p <0.0001) for the studies that reported reproducibility of CIMT and used the left and right CCA for CIMT in the meta-analysis. This was similar to the estimate for studies that reported reproducibility of CIMT and used one CCA for CIMT (0.084 mm, 95% CI [0.042, 0.126], p=0.0001). The summary estimate of the difference in CIMT for the two groups of studies that did not report the reproducibility of CIMT was higher, but not significantly different, for women with PCOS compared to controls. The average annual change in CIMT for women is estimated to be around 0.009[70] and 0.015 mm per year,[71] thus the summary mean difference corresponds to

about a seven year progression in CIMT. A large magnitude of difference in CIMT was detected in this meta-analysis despite including small studies of young women.

These results should be viewed in light of the significant heterogeneity across studies. As previously mentioned, these tests may have had low power. Nonetheless, heterogeneity could be due to between study differences in PCOS phenotypes, age, BMI, CVD risk factors and technical factors related to assessment of CIMT. Larger studies with a well-defined PCOS population using rigorous methodology may be required to draw a more robust conclusion. However, the evidence to date suggests women with PCOS are at greater risk of premature atherosclerosis that emphasizes the importance of screening and reducing CVD risk factors to prevent progression of CVD this high risk subgroup.

### 3.4.2 Strengths and Limitations of the Review

The limits of the search and the inclusion of only studies in the English language may have introduced possible publication bias in the meta-analysis. However only three non-English studies were excluded and we did not detect evidence of publication bias from the funnel plots or Egger's test. Another limitation was the heterogeneity that suggested the populations and CIMT measurements were not the same across studies. The heterogeneity was addressed by using the random effects model and grouping the studies according to quality of the CIMT measurement.

CIMT is a reproducible measure, but has within and between study variability due to random error and error from study participants and technicians. Larger variability of CIMT would decrease the reproducibility and require larger sample sizes to maintain adequate power. The subgroup analysis showed that the consistency of the estimates across studies increased as the quality of the CIMT measurement increased. The higher quality studies had consistent

CIMT estimates across studies and a more robust summary estimate. This is in contrast to the lower quality studies in which the estimates had more variation across studies. There were a few estimates with a wide CI that suggested lower precision in the estimate, and the summary estimate was much weaker and not significant at $p<0.05$. These observations demonstrate the importance of reporting quality control measures and describing the protocol and reproducibility of CIMT. A large portion of the studies did describe quality control measures for the CIMT measurement.[22-24,47,53-56,65,67,69]

Heterogeneity between studies could also be due to differences in the prevalence of CVD risk factors and PCOS phenotypes. The Rotterdam criterion adds an additional less severe phenotype to the diagnosis, which could increase heterogeneity and may lower the power of a study to detect a difference between PCOS and non PCOS participants. There is evidence that the prevalence of CVD risk factors vary by PCOS phenotypes.[72] Women with the classical definition of PCOS had a higher prevalence of one or more CVD risk factors (C-reactive protein, lipids, homocysteine) than ovulatory women with PCOS. Women with classical PCOS also had more abdominal obesity than ovulatory women with PCOS with similar BMI ($p<0.05$). On the other hand, nonhyperandrogenic PCOS women, which are included in the Rotterdam criteria and not the NIH criteria, had normal androgen levels and lower prevalence of insulin resistance and metabolic abnormalities than classical or ovulatory PCOS.

The meta-analysis is also vulnerable to limitations within each study that include their cross-sectional study designs and small sample sizes. Smaller studies may have lacked sufficient power to detect a difference in CIMT between cases and controls as the sample sizes of the studies ranged from 18 to 200 women with PCOS and 12 to 142 controls. Another limitation was the potential selection bias within the studies. The response rate of recruitment was not reported in any of the studies, which may lead to potential selection bias. In addition, there were differences in the average age and BMI between women with PCOS and controls in

some studies, but this was controlled for in all of the studies except three.[64,65,67] An analysis without these studies did not change the results.

The strength of this paper is that the meta-analysis was able to summarize results from the conflicting body of literature and increase statistical power by estimating a summary effect. Also, grouping studies by the quality of the CIMT measurement identified potential sources of heterogeneity and demonstrated the robustness of the results. This first meta-analysis to investigate CIMT between women with PCOS and controls was able to assess a reliable measure of subclinical atherosclerosis using studies limited with small sample size.

### 3.4.3 Comparison with Previous Research

Overall, this meta-analysis indicated women with PCOS had a 0.072 to 0.084 mm higher CIMT compared to controls. This is similar to studies showing women with PCOS are at higher risk of subclinical atherosclerosis as measured by CAC and FMD. Women with PCOS had higher prevalence of CAC (none versus any)[9-11] and more CAC (CAC <10 versus ≥10)[8] and significantly lower FMD[5,16,21,73] when compared to controls.

There are many mechanisms that may explain the increase in CIMT among women with PCOS. Studies suggested insulin,[68] total cholesterol and LDL cholesterol,[53,65] HDL cholesterol,[45] triglycerides,[65] CRP,[74] serum interleukin-18,[75] and abdominal obesity[68,74] were associated with CIMT in women with PCOS. Women with PCOS had higher insulin levels or insulin resistance compared to controls in all studies included in the meta-analysis except four.[13,22,23,53] This analysis showed women with PCOS have greater CIMT than controls, but this analysis cannot evaluate the influence of CVD risk factors that are strongly associated with PCOS and CIMT. Understanding the complexity of PCOS and the risk of CVD requires further classification of PCOS phenotypes and CVD risk factors in PCOS.

### 3.4.4 Conclusions

The findings from the first meta-analysis on subclinical atherosclerosis in women with PCOS show greater CIMT in women with PCOS when compared to controls. Heterogeneity was observed across studies, which may be due to that fact that PCOS is a common complex heterogeneous syndrome associated with CVD risk factors. The results showed greater variation in the CIMT estimates across studies as the quality of the CIMT measurement decreased, which could partially explain inconsistencies in the literature that could be improved by using standardized ultrasound protocols and reporting detailed methods for CIMT.

Identifying PCOS as a risk factor for CVD is difficult given the complexity of PCOS and the horizon for when CVD events occur with aging. To date, most studies have been conducted in young women, but the risk of CVD may not be evident until later in life. Large prospective studies with detailed PCOS phenotypic data and change in subclinical atherosclerosis are needed to provide a better estimate of the risk of CVD in women with PCOS.

In the absence of these studies, PCOS is accompanied by CVD risk factors that place these women at an increased risk of atherosclerosis. These findings enforce recommendations for screening and monitoring CVD risk factors in women with PCOS as endorsed by the Androgen Excess and PCOS society.[30] This is of important public health significance as it will allow for the early identification of hypertension, type 2 diabetes and premature atherosclerosis in this high risk population.

## 3.5 FUNDING AND ACKNOWLEDGEMENTS

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## 3.6 TABLES AND FIGURES FOR CHAPTER THREE

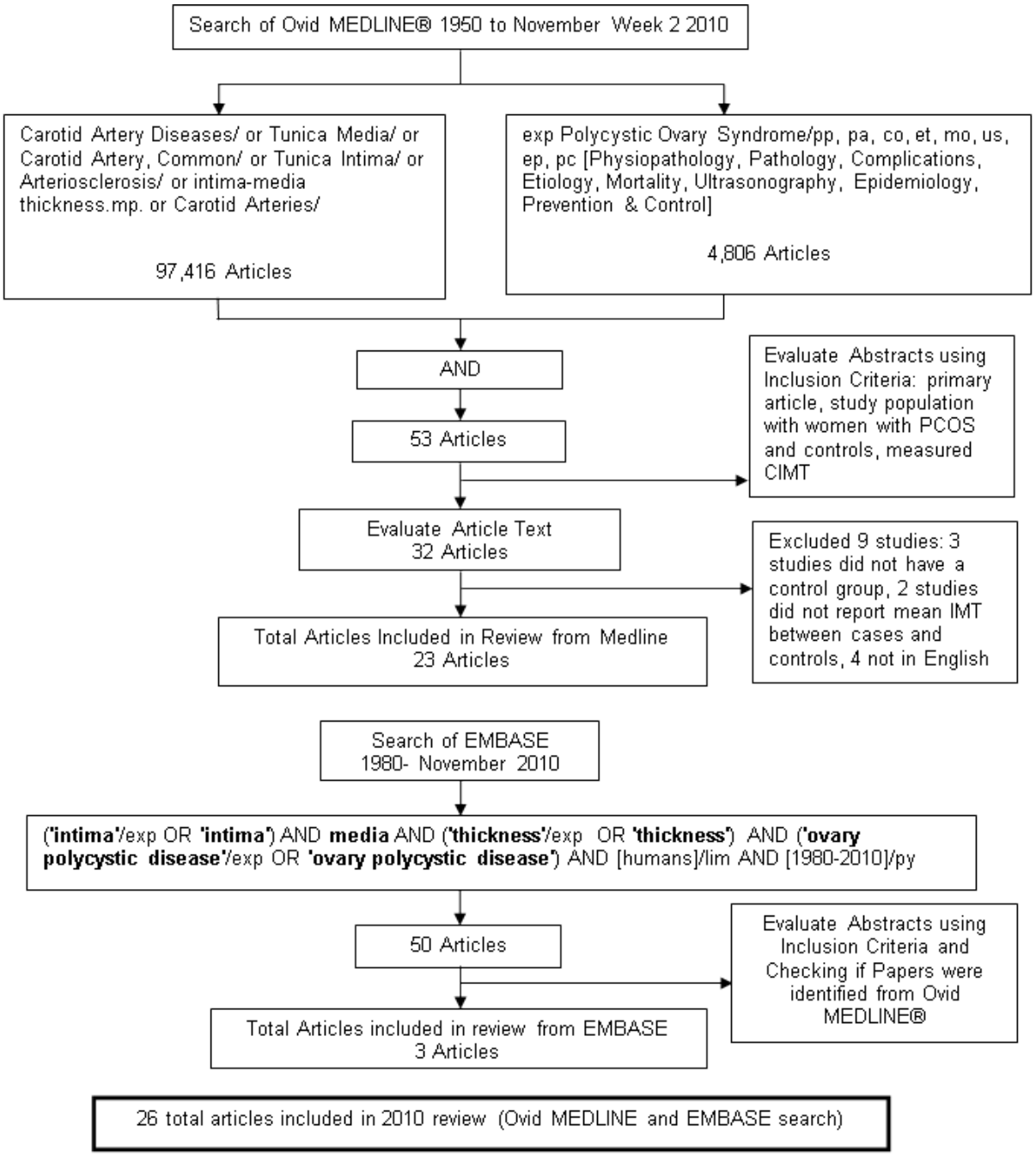
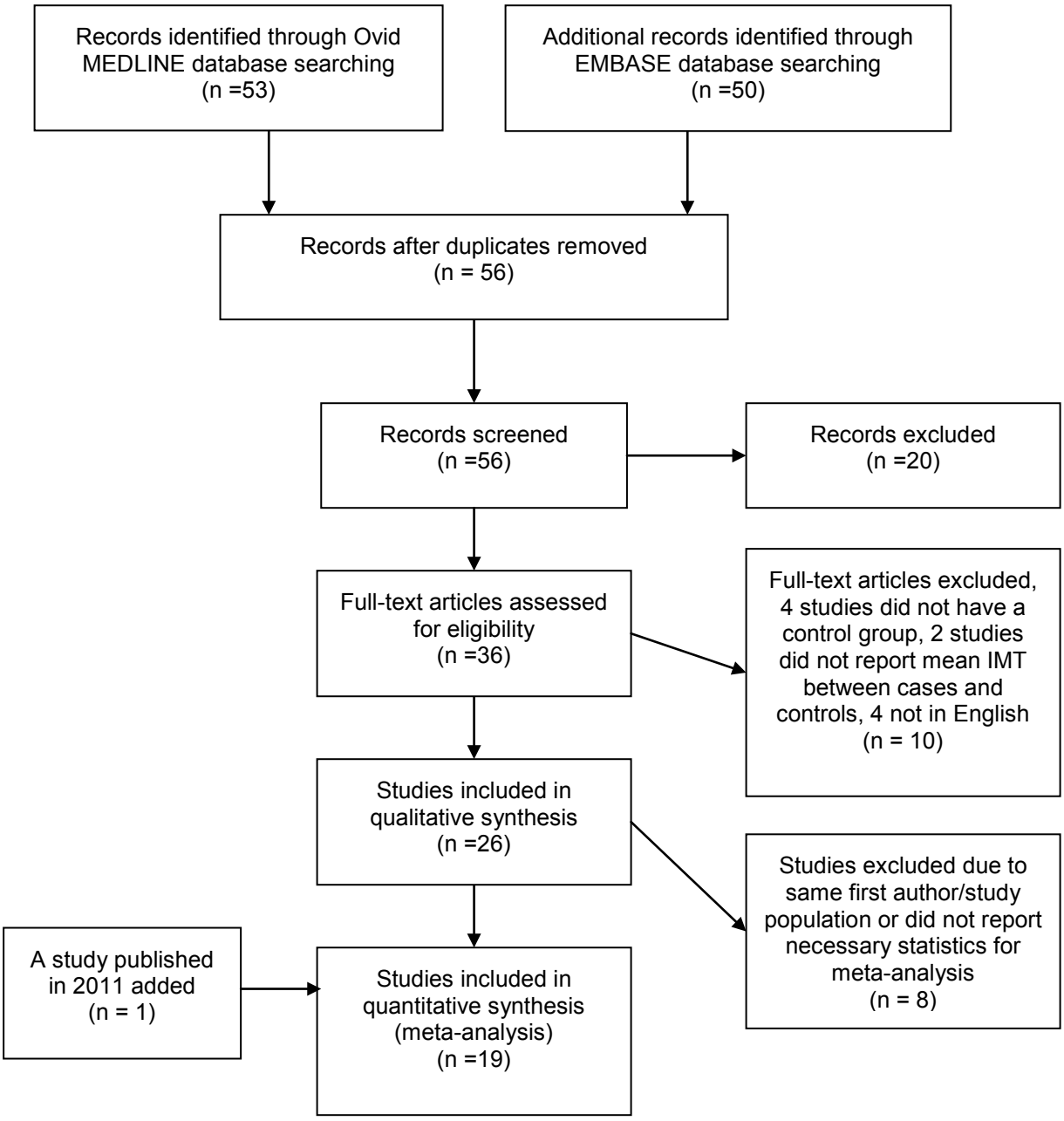



Figure 3.1 Diagram of the search strategy used to identify articles for the systematic literature review of carotid intima-media thickness (CIMT) in women with polycystic ovary syndrome and controls




*Modeled From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097.

Figure 3.2 PRISMA 2009 Flow Diagram

Table 3.1 Summary studies of CIMT in women with PCOS and controls for the qualitative review

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | p-value | CIMT protocol | Limitations | Reason for not including in meta-analysis |
|---|---|---|---|---|---|---|---|---|---|---|
| Guzick D, 1996 | Community | Age | 16 cases[a]<br>16 controls | 44.4±0.9[c]<br>43.9±1.3[c] | 32.7±2.3[c]<br>25.3±1.2[c] | 0.680±0.019[c]<br>0.630±0.012[c] | p=0.035 | Mean, left and right, near and far wall CCA, far wall bulb and ICA | Did not adjust for BMI (p=0.01). Hormone use was not addressed. Only 16 of the 38 eligible cases participated. | Same population as Talbott 2000 |
| Meyer C, 2005 | Community | NA | 100 cases[a]<br>20 controls | 32.7±1.8[c]<br>33.2±2.3[c] | 37.3±2.43[c]<br>36.7±1.28[c] | 0.55±0.01[c]<br>0.54±0.01[c] | p>0.05 | Mean, right, far wall CCA | | Did not report necessary statistics |
| Vryonidou A, 2005 | Patients seeking treatment for thyroid function and obesity | Age and BMI | 75 cases[a]<br>55 controls | 23.9±5.4<br>24.7±5.3 | 27.3±7.0<br>26.3±7.7 | 0.58 (0.42-0.80)[d]<br>0.47 (0.38-0.63)[d] | p<0.001 | Mean and Max, left and right, far wall CCA | Did not give standard deviation or error for CIMT or the exact p-value. Did not specify if they were taking hormones. | Did not report necessary statistics |
| Carmina E, 2006 | NA | Age and Weight | 50 cases[a]<br>50 controls | 25.2±1[c]<br>25.1±0.7[c] | 28.7±0.8[c]<br>28.5±0.5[c] | 0.50±0.01[c]<br>0.41±0.01[c] | p<0.01 | Mean, left and right, near and far walls CCA | Did not take into account hormone use. | Included Carmina 2009 |
| Cascella T, 2006 | NA | Age and BMI | 50 cases[b]<br>50 controls | 21.9±2.7<br>22.2±2.8 | 24.6±2.5<br>24.4±2.8 | 0.50±0.07<br>0.40±0.05 | p<0.001 | Mean, left and right, far wall CCA | Did not mention if controls were off hormones. | Included Cascella 2008 |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]Data expressed as median (minimum-maximum); NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

Table 3.1 Continued

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | p-value | CIMT protocol | Limitations | Rational for not including in meta-analysis |
|---|---|---|---|---|---|---|---|---|---|---|
| Erdogan M, 2008 | NA | NA | 68 cases[b] | 24.3±5.4 | 24.4±5.4 | Right 0.42±0.50<br>Left 0.43±0.56 | p>0.05<br>p>0.05 | NA | No description of the CIMT definition and protocol. | Included Erdogan 2009 |
| | | | 26 controls | 26.4±5.7 | 23.4±5.0 | Right 0.44±0.56<br>Left 0.44±0.77 | | | | |
| Adali E, 2010 | Outpatient clinic | Age | 24 overweight or obese cases[b] | 26.7±2.2 | 29.7±3.6 | 0.04±0.01 | p>0.05 | Mean of left and right CCA , average of 5 measure-ments | Split up cases so they might be underpowered. Did not mention smoking status. Obese cases BMI significantly higher than lean cases and controls (P<0.05). | Unusual CIMT values |
| | | | 26 nonobese cases[b] | 24.7±2.9 | 24.4±4.2 | 0.03±0.01 | | | | |
| | | | 25 controls | 25.0±2.3 | 23.9±4.0 | 0.03±0.01 | | | | |
| Pamuk 2010 | Healthy hospital staff | Age and BMI | 35 cases[b] | 26 (18-35)[d] | 29.7 (23.9-34.4)[d] | 0.52 (0.45-0.72)[d] | 0.51 | Right and left CCA, average of 5 measure-ments | | Did not report necessary statistics |
| | | | 31 controls | 27 (18-33)[d] | 28.4 (23.1-33.8)[d] | 0.49 (0.40-0.71)[d] | | | | |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]Data expressed as median (minimum-maximum); NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

Table 3.2 Summary of studies of CIMT in women with PCOS and controls for the meta-analysis

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | | p-value | Segment(s) used to measure CIMT | CIMT protocol |
|---|---|---|---|---|---|---|---|---|---|---|
| Talbott E, 2000 | Community | Age and BMI | 125 cases[a] | 37.5±6.2[c] | 30.1±0.7[c] | Age-BMI Adjusted (CI) | | | CCA, Bif, ICA | Mean, left and right, near and far walls, Intra-class correlation coefficient 0.86 |
| | | | | | | Overall (n=125) | 0.7 (0.68-0.73) | p=0.299 | | |
| | | | | | | 30-44 y (n=78) | 0.65 (0.62-0.69) | p=0.565 | | |
| | | | | | | ≥ 45 y (n=47) | 0.77 (0.74-0.81) | p=0.005 | | |
| | | | 142 controls | 39.0±6.2[c] | 26.5±0.5[c] | Overall (n=142) | 0.67 (0.65-0.69) | | | |
| | | | | | | 30-44 y (n=82) | 0.64 (0.61-0.67) | | | |
| | | | | | | ≥ 45 y (n=60) | 0.71 (0.68-0.75) | | | |
| Lakhani K, 2004 | Staff members | Age and BMI | 19 PCOS[b] | 29.2±4.0 | 31.3±8.2 | 0.54±0.11 | 0.53±0.09[d] | p=0.006, p=0.034[d] | CCA, Bif | Mean, right side, CCA and Bif reported separately, intra-observer coefficient of variation (CV) 8% |
| | | | 12 PCO | 27.7±4.0 | 22.5±3.8 | 0.51±0.18 | 0.50±0.15[d] | p=0.038, p=0.841[d] | | |
| | | | 12 controls | 27.5±4.0 | 24.2±3.4 | 0.40±0.09 | 0.44±0.09[d] | | | |
| Orio F, 2004 | NA | Age and BMI | 30 cases[b] | 22.2±2.5 | 22.4±2.1 | 0.53±0.09[e] | | p<0.05 | CCA | Mean, right and left, far wall, intra-observer CV 7% and inter-observer CV12%. |
| | | | 30 controls | 22.6±2.3 | 22.1±1.8 | 0.39±0.08[e] | | | | |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]CIMT adjusted for age, systolic and diastolic BP, BMI, cholesterol and insulin; [e]Adjusted for age, BMI, waist to hip ratio, smoking status, glucose and insulin levels, FAI, lipid profile, Hcy concentrations, and SBP and DBP; [f]Androgen Excess Society (AES) PCOS criteria; *Randomized clinical trial; NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

Table 3.2 Continued

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | p-value | Segment(s) used to measure CIMT | CIMT protocol |
|---|---|---|---|---|---|---|---|---|---|
| Vural B, 2005 | Medical students and nurses | Age | 43 cases[b]<br>43 controls | 21.4±1.8<br>20.8±2.2 | 23.4±4.7<br>21.5±3 | 0.75±0.11<br>0.61±0.11 | p<0.001 | CCA | Left and right, near and far walls, intra-observer error <0.03 mm |
| Alexan-draki K, 2006 | Doctors and medical students | Age and BMI | 27 cases[a]<br>27 controls | 25.4±0.8[c]<br>27.3±0.8[c] | 27.42±1.1[c]<br>25.05±1.2[c] | 0.49±0.01[c]<br>0.51±0.02[c] | p=0.19 | CCA, Bif, ICA | Mean, left and right, far wall , one reader |
| Luque-Ramirez M, 2007 | 12 patients for obesity problems, 8 healthy controls | | 40 cases[a]<br>20 controls | 24.5±5.8<br>27.2±6.8 | 29.4±6.3<br>28.2±6.9 | 0.41±0.11<br>0.33±0.08 | p=0.005 | CCA | Left, far wall, intra-observer CV 10.8% |
| Cascella T, 2008 | NA | Age and BMI | 200 cases[b]<br>100 controls | 24.6±3.2<br>24.0±2.8 | 28.5±2.8<br>28.8±2.7 | 0.46±0.16<br>0.38±0.09 | p<0.001 | CCA | Mean, left and right, far wall, intra-observer CV 7.0% |
| Costa L, 2008 | NA | | 57 cases[b]<br>37 controls | 25.5±5.3<br>26.6±5.4 | 27.6±5.8<br>26.7±4.9 | 0.52±0.08<br>0.53±0.08 | p=0.35 | CCA | Mean, left and right, far wall, one reader |
| Heutling D, 2008* | Public advertising | | 83 cases[b]<br>39 controls | 24.8±4.7<br>27.8±5.6 | 30.4±5.9<br>29.1±4.8 | 0.48±0.07<br>0.42±0.05 | p<0.001 | CCA | Mean, left and right, far wall, intra-observer CV 6.8% |
| Karadeniz M, 2008 | Patients | Age | Cases[b] HOMA-IR>1.75 (n=37) | 23.8±5.5 | 25.6±5.6 | Right: 0.41±0.05<br>Left: 0.43±0.06 | p>0.05<br>p>0.05 | CCA | NA |
| | | | Cases[b] HOMA-IR<1.75 (n=21) | 24.9±5.1 | 22.2±4.1 | Right: 0.43±0.05<br>Left: 0.45±0.06 | p>0.05<br>p>0.05 | | |
| | | | 25 controls | 27.2±4.2 | 23.4±5.2 | Right: 0.44±0.05<br>Left: 0.44±0.05 | | | |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]CIMT adjusted for age, systolic and diastolic BP, BMI, cholesterol and insulin; [e]Adjusted for age, BMI, waist to hip ratio, smoking status, glucose and insulin levels, FAI, lipid profile, Hcy concentrations, and SBP and DBP; [f]Androgen Excess Society (AES) PCOS criteria; *Randomized clinical trial; NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

Table 3.2 Continued

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | p-value | Segment(s) used to measure CIMT | CIMT protocol |
|---|---|---|---|---|---|---|---|---|---|
| Saha S, 2008 | Staff members | | 30 cases[a] | 26.1±4.2 | 25.8±4.6 | 0.63±0.19 | p<0.001 | CCA, Bif, ICA | Mean, left and right, NS |
| | | | 30 controls | 28.7±7.1 | 22.0±3.0 | 0.44±0.05 | | | |
| Trakakis E, 2008 | Nurses and medical students | | 53 cases[b] | 26.1±5.5 | 28.7±7.1 | Right: 0.67±0.15<br>Left: 0.68±0.13 | p<0.0001<br>p<0.0001 | CCA, ICA | Mean IMT, left and right, near and far wall |
| | | | 53 controls | 25.4±4.7 | 28.7±7.1 | Right: 0.46±0.16<br>Left: 0.42±0.16 | | | |
| Arikan S, 2009 | Staff and medical students | Age and BMI | 39 cases[b] | 22.8±5.5 | 21.5±6.5 | 0.45±0.82 | p>0.05 | CCA | Mean, left and right, far wall, one technician |
| | | | 30 controls | 24.6±4.2 | 20.9±6.0 | 0.44±0.11 | | | |
| Carmina E, 2009 | Family members of staff | Age and weight | 95 cases[f] | 24.2±3 | 27.6±5.8 | 0.61±0.18 | p< 0.01 | CCA | Mean average of 10 measurements of left and right far wall, intra-observer CV <7.0% |
| | | | 90 controls | 23.9±3 | 27.5±3 | 0.53±0.15 | | | |
| Ciccone M, 2009 | NA | Age | 29 cases[b] | 22.0±3.8 | 26.3±4.5 | 0.651±0.59 | p>0.05 | CCA, ICA, Bif | Mean of average of 3 measures from right and left, plaque free segments, one technician |
| | | | 26 controls | 22.0±3.8 | 20.5±1.6 | 0.637±0.133 | | | |
| Soares G, 2009 | Basic health clinic | Age and BMI | 40 cases[b] | 24.5±3.8 | 22.7±3.3 | 0.44±0.10 | p=0.41 | CCA | Mean of average of 4 measures of right CCA |
| | | | 50 controls | 24.5±5.1 | 23.1±3.2 | 0.42±0.09 | | | |
| Erdogan M, 2009 | Outpatient clinic | Age and BMI | 88 cases[b] | 24.1±1.32 | 24.4±4.1 | Right: 0.74±0.59<br>Left: 0.73±0.80 | p>0.05 | CCA | NA |
| | | | 119 controls | 25.0±2.1 | 23.5±4.1 | Right: 0.74±0.61<br>Left: 0.74±0.60 | | | |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]CIMT adjusted for age, systolic and diastolic BP, BMI, cholesterol and insulin; [e]Adjusted for age, BMI, waist to hip ratio, smoking status, glucose and insulin levels, FAI, lipid profile, Hcy concentrations, and SBP and DBP; [f]Androgen Excess Society (AES) PCOS criteria; *Randomized clinical trial; NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

Table 3.2 Continued

| First Author, Date | Control Population | Controlled for Age and/or BMI | N | Age±SD | BMI±SD | Mean CIMT±SD (mm) | p-value | Segment(s) used to measure CIMT | CIMT protocol |
|---|---|---|---|---|---|---|---|---|---|
| Ketel I, 2010 | Clinic, local newspaper advertisements | Age and weight | 22 lean cases[b] | 28.6±4.5 | 22.0±2.2 | 0.53±0.08 | p>0.05 | CCA | Right side, mean of 3 measurements |
| | | | 18 obese cases[b] | 30.3±4.2 | 36.2±5.9 | 0.56±0.17 | | | |
| | | | 17 lean controls | 27.7±5.3 | 22.2±1.7 | 0.48±0.07 | | | |
| | | | 13 obese controls | 28.6±5.3 | 40.5±7.0 | 0.60±0.11 | | | |
| Pepene C, 2011 | NA | Age | 64 cases[f] | 28.6±5.4[c] | 29.9±0.8[c] | 0.57±0.02[c] | p=0.323 | CCA, Bif, ICA | Single max CIMT, left and right, one tech, CV 5% |
| | | | 20 controls | 28.6±5.5[c] | 26.3±1.3[c] | 0.64±0.06[c] | | | |

[a]NIH PCOS Criteria; [b]Rotterdam PCOS Criteria; [c]Data expressed as Standard Error (SE); [d]CIMT adjusted for age, systolic and diastolic BP, BMI, cholesterol and insulin; [e]Adjusted for age, BMI, waist to hip ratio, smoking status, glucose and insulin levels, FAI, lipid profile, Hcy concentrations, and SBP and DBP; [f]Androgen Excess Society (AES) PCOS criteria; *Randomized clinical trial; NA, Not Addressed; CCA, Common carotid artery; Bif, Carotid bifurcation; ICA, Internal carotid artery; P values are cases versus controls

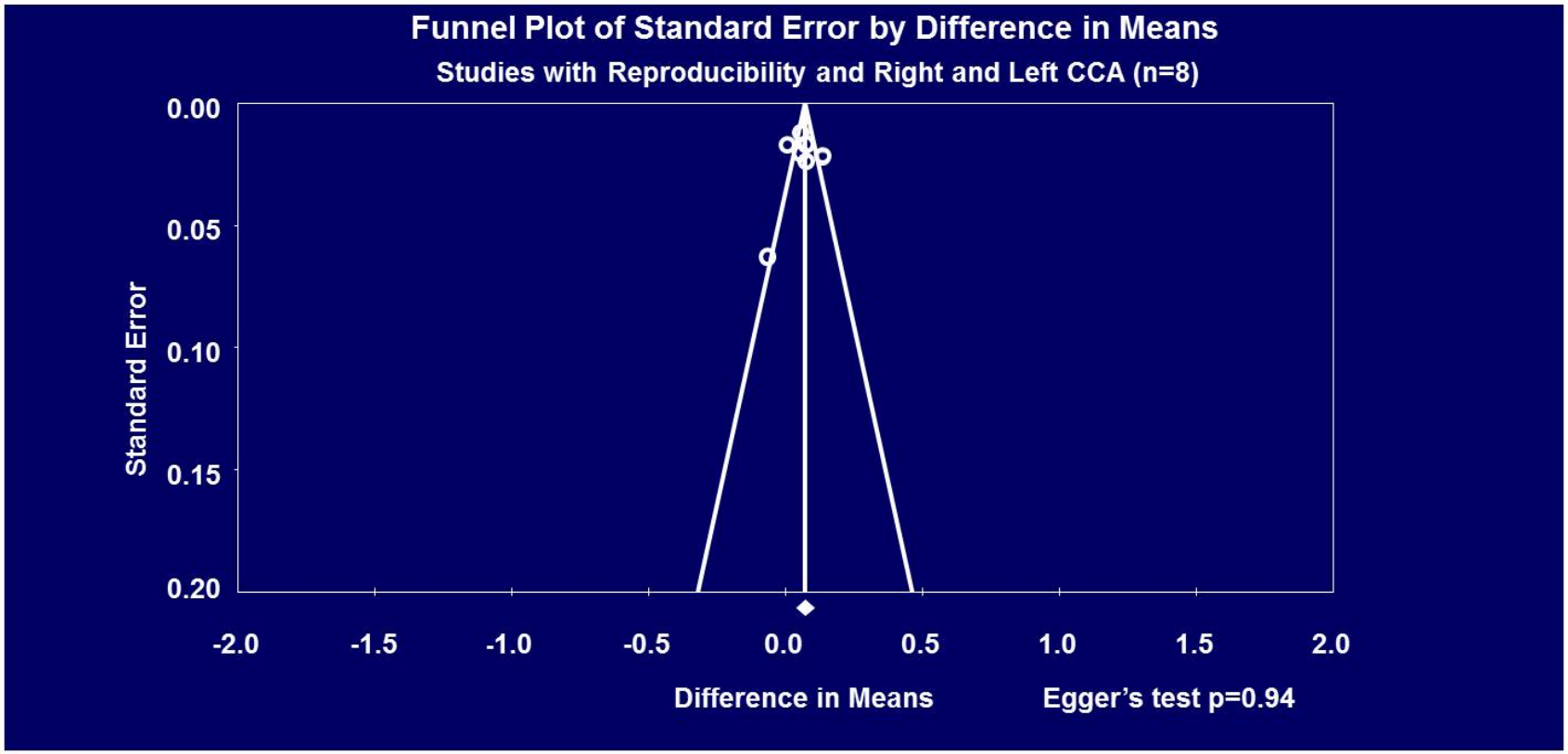



Figure 3.3 Funnel plot to assess publication bias among the highest quality studies: random effects model

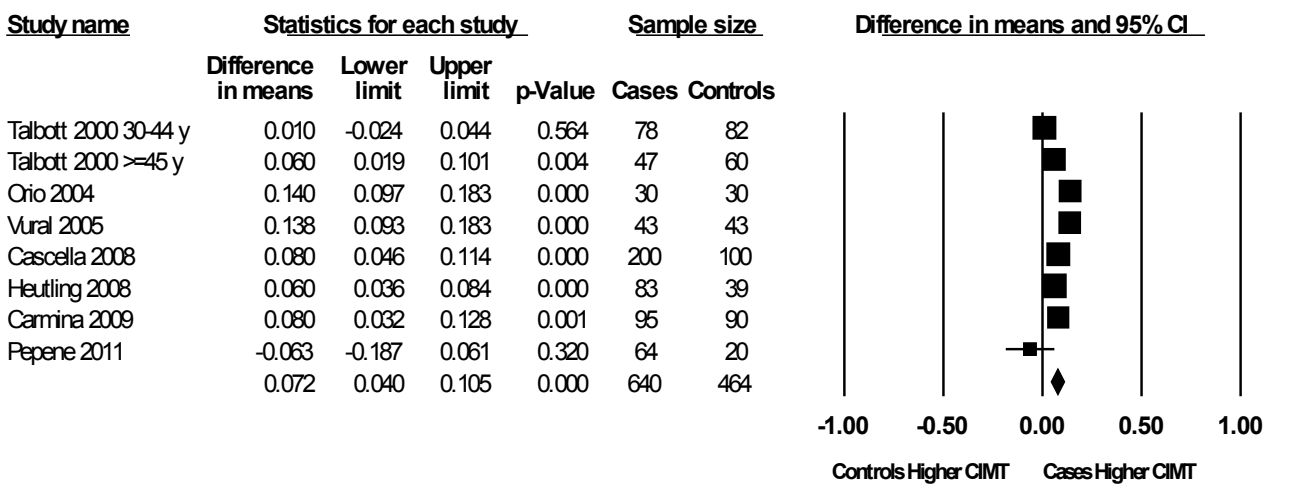



Figure 3.4 Forest plot of studies that reported a reproducibility statistic and used the left and right CCA for CIMT

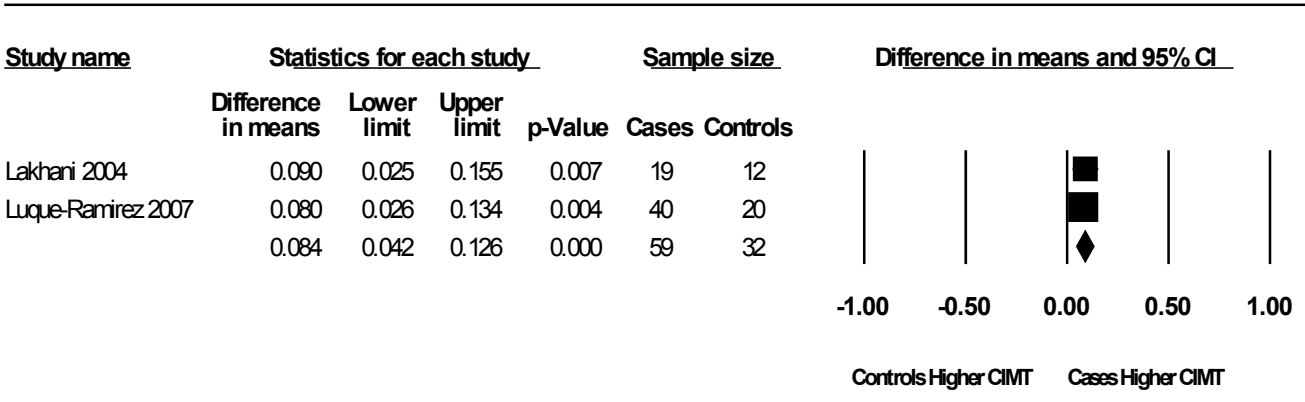



Figure 3.5 Forest plot of studies that reported a reproducibility statistic and used the left and right CCA for CIMT

| Study name | Statistics for each study | | | | Sample size | | Difference in means and 95% CI |
|---|---|---|---|---|---|---|---|
| | Difference in means | Lower limit | Upper limit | p-Value | Cases | Controls | |
| Alexandraki 2006 | -0.020 | -0.050 | 0.010 | 0.184 | 27 | 27 | |
| Costa 2008 | -0.010 | -0.043 | 0.023 | 0.554 | 57 | 37 | |
| Saha 2008 | 0.190 | 0.120 | 0.260 | 0.000 | 30 | 30 | |
| Arikan 2009 | 0.010 | -0.286 | 0.306 | 0.947 | 39 | 30 | |
| Ciccone 2009 | 0.014 | -0.218 | 0.246 | 0.906 | 29 | 26 | |
| | 0.041 | -0.038 | 0.120 | 0.310 | 182 | 150 | |

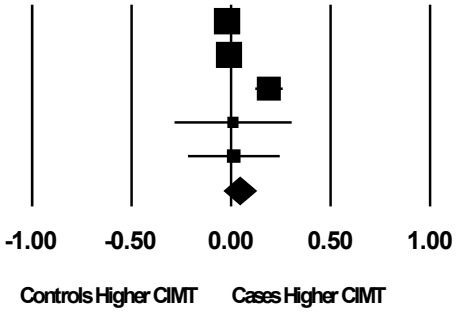



Figure 3.6 Forest plot of studies that did not report a reproducibility statistic and used the left and right CCA for CIMT

| Study name | Statistics for each study | | | | Sample size | | Difference in means and 95% CI |
|---|---|---|---|---|---|---|---|
| | Difference in means | Lower limit | Upper limit | p-Value | Cases | Controls | |
| Karadeniz 2008 | -0.003 | -0.031 | 0.025 | 0.835 | 21 | 25 | |
| Trakakis 2008 | 0.211 | 0.152 | 0.271 | 0.000 | 53 | 53 | |
| Soares 2009 | 0.020 | -0.019 | 0.059 | 0.319 | 40 | 50 | |
| Erdogan 2009 | -0.002 | -0.168 | 0.164 | 0.981 | 88 | 119 | |
| Ketel 2010 Lean | 0.050 | 0.002 | 0.098 | 0.041 | 22 | 17 | |
| Ketel 2010 Obese | -0.040 | -0.146 | 0.066 | 0.458 | 18 | 13 | |
| | 0.045 | -0.020 | 0.111 | 0.173 | 242 | 277 | |

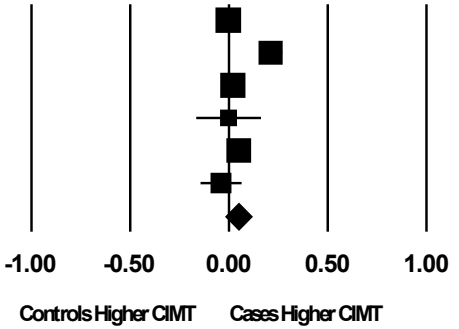



Figure 3.7 Forest plot of studies that did not report a reproducibility statistic and used the left and right CCA for CIMT

Table 3.3 PCOS and CIMT meta-analysis results for random effects models by quality of CIMT measurement

| | Random Effects Model | Number of Studies | Point Estimate | SE | 95% CI | P-value | Heterogeneity* $\chi^2$ (p-value) | Heterogeneity* $I^2$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Studies that reported reproducibility and used the right and left CCA for CIMT | 8 | 0.072 | 0.017 | 0.040, 0.105 | <0.0001 | 36.818 (<0.0001) | 80.988 |
| 2 | Studies that reported reproducibility and used one CCA for CIMT | 2 | 0.084 | 0.021 | 0.042, 0.126 | <0.0001 | 0.054 (0.817) | 0.000 |
| 3 | Studies that did not report reproducibility and used the right and left CCA for CIMT | 5 | 0.041 | 0.040 | -0.038, 0.120 | 0.310 | 30.113 (<0.0001) | 86.717 |
| 4 | Studies that did not report reproducibility and used one CCA for CIMT | 6 | 0.045 | 0.033 | -0.020, 0.111 | 0.173 | 43.575 (<0.0001) | 88.526 |
| | All Studies | 21 | 0.059 | 0.014 | 0.031, 0.088 | <0.0001 | 144.804 (<0.0001) | 86.188 |

SE: Standard Error; CI: Confidence Interval; *heterogeneity estimates based on fixed effects model, $\chi^2$ from Q-value

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# 4.0 COMPLEMENT PROTEIN C3 AND CORONARY ARTERY CALCIUM IN MIDDLE-AGED WOMEN WITH POLYCYSTIC OVARY SYNDROME AND CONTROLS

## 4.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) affects 6-10% of women in the United States and is the most common reproductive endocrine disorder.[1,2] Women with PCOS experience acne, excessive hair, weight gain and irregular periods. These women also have an increase in cardiovascular disease (CVD) risk factors including insulin resistance,[3] dyslipidemia,[4] abdominal obesity,[5] type 2 diabetes[6] and inflammation.[7] There is limited evidence of an increased risk in CVD events, but studies show women with PCOS have an increased risk of subclinical atherosclerosis as measured by coronary artery calcification (CAC),[8-11] and adverse vascular structural and functional changes measured by carotid artery intima-media thickness[12-19] and flow mediated dilation[5,20-22] when compared to controls.

The exact roles of PCOS related factors involved in atherosclerosis are inadequately defined and may have long-term effects on the progression of atherosclerotic lesions. Recent studies suggest that PCOS is a low-grade inflammatory state, which is concerning since atherosclerosis is classified as a vascular inflammatory disease. The process involves inflammatory components triggering, initiating and promoting atherosclerosis.[23-25] Pro-inflammatory response proteins have been found in atherosclerotic lesions[26-28] and plaques.[29]

The inflammatory markers include those of the innate immune system, which provides immediate defense against pathogens in part through the complement cascade. The complement system contains over 30 complement proteins; however, complement protein C3 (C3) is of particular interest because it is the central component in the complement cascade. The classical, alternative and mannose-binding lectin pathways of the inflammatory response converge at C3, which cleaves C3 and elicits the inflammatory response. The inflammatory response is essential to protect the body for disease and injury, but has the potential to promote vascular damage under certain circumstances.[23,30] C3 is a proposed inflammatory marker of atherosclerosis as complement levels have been elevated in people with CVD[31-33] and C3 has been associated with tissue damage at the site of myocardial infarctions.[34]

Currently, the association of C3 and subclinical measures of atherosclerosis in the general population is unknown. The association of C3 with subclinical CVD measures has been investigated in women with systemic lupus erythematosus (SLE), which is also a high CVD risk population. High circulating C3 levels in young women with SLE were associated with subclinical CVD measures including an increased prevalence of coronary artery calcium (CAC)[35] and vascular stiffness measured by pulse wave velocity (PWV).[36,37]

No investigation has been completed of the association between C3, CVD risk factors, and atherosclerosis in middle-aged women with PCOS and controls. The aim of this study was to determine whether circulating serum C3 levels are higher in women with PCOS compared to non-PCOS controls, and whether C3 levels are associated with traditional CVD risk factors and CAC in women with PCOS and non-PCOS controls.

## 4.2 METHODS

### 4.2.1 Study Population

This study is based on the third phase of the Cardiovascular Health and Risk Measurement Study (CHARM III) that occurred between 2001 and 2003. There were a total of 319 women (151 women with PCOS and 168 controls) in CHARM III; however, this present analysis includes 132 women with PCOS and 155 controls aged 35-62 years old in which stored serum was available in 2010. The CHARM recruitment and methodology has previously been described.[11,38] Briefly, investigators identified women aged 19-55 years old diagnosed with PCOS between 1970 and 1993 through medical records in the Division of Reproductive Endocrinology at Magee-Women's Hospital (Pittsburgh, PA). Investigators used NIH criteria to define PCOS.[39] Investigators matched PCOS cases to neighborhood controls by age (±5 years) and race using voter's registration tapes and the Cole's Cross Reference Directory of Households from 1993. The University of Pittsburgh institutional review board approved the protocols and all participants gave consent before enrolling.

### 4.2.2 Data Collection

The clinical visit and assessments of participant characteristics have been previously described.[8,11,38] In brief, investigators collected medical, surgical, menstrual and reproductive history, medication use, lifestyle, anthropometric measurements, blood pressure and serum concentrations of total cholesterol, HDL-C, LDL-C, triglycerides, fasting glucose and insulin, and hormones that included sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone, progesterone and estradiol. The quantitative

insulin sensitivity check index (QUICKI) was also calculated (QUICKI= 1/[log fasting insulin concentration (μU/mL) + log glucose concentration (mg/dL)]).[40] QUICKI is a measure of insulin sensitivity where lower values indicate insulin resistance.

Blood serum samples were frozen during CHARM III in 2001-2003 and stored at the University of Pittsburgh Graduate School of Public Health. In 2010, Quest Diagnostics (Pittsburgh, PA) collected 287 stored serum samples and applied an immunoturbidimetric assay (C3c Serum Complement Assay, ID 44859W) with a Roche Integra System to measure C3 levels. The reference range for C3 from the Quest laboratory was 90-180 mg/dL. Quality control measures for this assay include a monthly audit comparing data bias and precision against other Quest laboratories operating the same platform and a guarantee through regulated College of American Pathologists (CAP) and New York State Proficiency.

### 4.2.3 Coronary Artery Calcium Assessment

The participants underwent an electron beam computed tomography (EBCT) of the heart and aorta to measure coronary artery calcification at the University of Pittsburgh Medical Center Preventive Heart Care Center. Methods have been previously described.[11] Briefly, the radiology and computer tomography technicians (RT/CT) were blinded to case control status and performed one scan per participant using the Imatron C-150 Ultrafast CT scanner (Imatron, South San Francisco, CA). The RT/CT technician scanned 30-40 contiguous images 3 mm from the aortic root to the apex of the heart with a 100 millisecond exposure time. Images were taken at the same phase of cardiac cycle, approximately 60% of the R-R interval.

Coronary calcium scores were computed using the base value region of interest computer software program (AccuImage, Diagnostics Corp., San Francisco, CA). Calcification was defined as pixels greater than 130 Houndsfield units and 1 $mm^2$ within an operator-defined

region of interest (ROI) in each 3 mm thick image. The Agatston method was used to calculate the calcium score for each ROI by multiplying the area of significant pixels by a grade number (1-4) indicative of the peak computerized tomography number (Hounsfield unit).[41] The individual ROI's were summed for a total coronary calcium score.

### 4.2.4 Statistical Analysis

This cross-sectional analysis of serum C3 levels and CAC contained 132 women with PCOS and 155 controls aged 35-62 years old. To account for the original age-matched design, the descriptive and correlation analyses were performed stratified by age groups <45 years old and ≥45 years old, and the logistic regression models were adjusted for age. Participant characteristics were calculated using descriptive statistics for women with PCOS and controls, and were compared using Chi-square tests or Fisher's exact test for categorical data and independent t-tests for normally distributed continuous variables or the Mann-Whitney U test for non-normally distributed variables. Pearson correlations were used to determine associations between C3 and CVD risk factors in women with PCOS and controls. Women using hormone replacement therapy or oral contraceptives were excluded when analyzing hormone levels so the results were not influenced by hormone use.

The association between C3 and CAC was determined by logistic regression analyses of CAC as a binary (presence of CAC Agatston score ≤0 vs. >0) as well as an ordinal variable (three group CAC categories) adjusting for PCOS status, age and significant CVD risk factors. Three categories of CAC: 0, 1-10, and ≥11 were created based from published guidelines for five categories of CAC (0, 1-10, 11-100, 101-400, 401+).[42] The last three groups were combined since few subjects had a CAC score greater than 100. To address the original age-

matched design, a sensitivity analysis was conducted using conditional logistic regression of the presence of CAC (Agatston score ≤0 vs. >0) with the 47 original age-matched pairs.

For all regression analyses, insulin and glucose levels were categorized into quartiles and C3 was expressed as a unit of 10 mg/dL. All participant factors with a significant univariate association of $p<0.20$ with CAC were evaluated in a forward stepwise method for the multivariable regression models. All first order interactions with C3 and case control status were tested. The test of parallel lines was performed for the ordinal logistic regression analyses to check the proportional odds assumption that the slope coefficients are the same across pairs of CAC groups.

Variables were presented as means±standard deviations or medians (inter-quartile range), odds ratios (OR) and 95% confidence intervals (CI). The level of statistical significance was a 2-sided p-value of <0.05. All analyses were done using PASW (version 18; IBM SPSS Inc., Chicago, IL, USA).

## 4.3 RESULTS

There were 55 women with PCOS and 39 controls that were <45 years old, and 77 women with PCOS and 116 controls that were ≥45 years old. In women <45 years old, women with PCOS and controls did not differ with respect to age (41.96±2.09 compared to 42.00±2.25 years old, p=0.92, respectively) and BMI (31.41±7.13 kg/m$^2$ compared to 29.23±6.61 kg/m$^2$, p=0.14, respectively) (Table 4.1). However in women ≥45 years old, women with PCOS were significantly younger than controls (50.44±3.79 compared to 51.75±4.21years old, p=0.03, respectively) and had a higher BMI than controls (31.53±7.91 kg/m$^2$ compared to 28.34±5.94 kg/m$^2$, p=0.002, respectively). Women with PCOS had significantly larger waist-to-hip ratio and

fasting insulin compared to controls among women <45 years. Women with PCOS ≥45 years old had significantly higher BMI, waist circumference, waist-to-hip ratio, fasting insulin, triglycerides and lower age, QUICKI and HDLc compared to controls. C3 was higher, but not significantly different, in women with PCOS than controls <45 years old (171.20±36.87 mg/dL compared to 168.79±35.11 mg/dL, p= 0.76), and in women with PCOS than controls ≥45 years old (175.12±39.85 mg/dL compared to 169.06±36.81 mg/dL, p= 0.28).

Significant differences were seen in levels of total testosterone and SHBG in women <45 years old and in SHBG, luteinizing hormone, FSH in women ≥45 years old, excluding women currently using HRT or OCs (Table 4.2). In women <45 years old, women with PCOS had higher total testosterone and lower SHBG than controls ($p<0.05$). In women ≥45 years old, women with PCOS had had lower SHBG, LH, and FSH than controls ($p<0.05$).

Women with PCOS and controls were similar with respect to categorical characteristics in women <45 years old; however in women ≥45 years old, women with PCOS were more obese (BMI≥30), and had more type 2 diabetics and fewer African American women compared to controls (Table 4.3). The presence of CAC (>0), in CAC 10+ or in the CAC groups did not differ between women with PCOS and controls in women <45 years old (Table 4.4). Conversely in women ≥45 years old, women with PCOS had more CAC >0 (64.9% versus 44.0%, p=0.004), CAC 10+ (35.1% versus 12.1%, $p<0.001$) and more women in higher CAC groups ($p<0.0001$) compared to controls.

There were a few differences in C3 levels with respect to categorical variables within the age and PCOS and control subgroups (data not shown). In controls <45 years old, the women currently using oral contraceptives had a higher C3 than the non-users, p=0.006. In women with PCOS ≥45 years old, the thirteen type 2 diabetics had higher C3 than the non-diabetics, p=0.002. In controls ≥45 years old, the African Americas had higher C3 than the Whites, p=0.03, and the three type 2 diabetics had higher C3 than the non-diabetics p=0.007.

Regardless of age groups or PCOS status, C3 positively correlated with BMI, waist circumference, waist-to-hip ratio, insulin, triglycerides and negatively correlated with QUICKI (all $p<0.01$) (Table 4.5). Except in controls <45 years old, C3 positively correlated with glucose ($p<0.01$) and negatively with HDLc ($p<0.01$, $p=0.05$ in controls ≥45 years old). C3 positively correlated with LDLc in women with PCOS and controls <45 years old ($p<0.01$) and with SBP in women with PCOS and controls ≥45 years old ($p<0.01$). C3 negatively correlated with SHBG in all groups ($p<0.02$) (Table 4.6). Estradiol negatively correlated with C3 in controls <45 years old ($r=-0.25$, $p=0.04$) and FSH positively correlated with C3 in women with PCOS <45 years old ($r=0.29$, $p=0.04$).

For the logistic and ordinal regression analysis, the significant variables associated with CAC included case control status, age, C3, BMI, insulin quartiles and African American race. In the logistic regression analysis of the presence of CAC, a 10 mg/dL unit increase in C3 was associated with the presence of CAC after adjusting for case control status and age (OR 1.39, 95% CI [1.27, 1.52], $p<0.0001$), when additionally adjusting for insulin quartiles (OR=1.26, 95% CI [1.14, 1.39], $p=0.011$) or was borderline significant when adjusting for BMI (OR=1.14, 95% CI [1.03, 1.27], $p=0.072$) (Table 4.7). C3 was significantly related to the presence of CAC (OR=1.12, 95% CI [1.00, 1.25], $p=0.049$) in the fully adjusted model that included age, PCOS status, BMI, insulin quartiles, and African American race.

In ordinal logistic regression analysis adjusting for case control status and age, the expected odds of a higher CAC category for a ten unit increase in C3 (mg/dL) was 1.91 (95% CI [1.35, 2.71], $p<0.0001$), and remained significant after additionally adjusting for insulin quartiles (OR=1.14 (95% CI [1.08, 1.20], $p<0.0001$) or BMI (OR=1.09 (95% CI [1.03, 1.15], $p=0.003$) (Table 4.8). The association between C3 and CAC was attenuated but borderline significant (OR=1.06 (95% CI [0.99, 1.12], $p=0.082$) in the fully adjusted model that included age, PCOS status, BMI, insulin quartiles, and African American race.

There were no significant interactions between C3 and PCOS status with any variables in the binary and ordinal regression models. In the ordinal logistic regression analyses, the test of parallel lines was not significant, which indicated the proportional odds assumption was met. All categorical variables were assessed as possible confounders in the fully adjusted regression models. The only significant variable after adjusting for age and case control status was type 2 diabetes. C3 remained significantly associated with the presence of CAC after adjusting for age, case control status, BMI, African American race, and type 2 diabetes (OR=1.15, 95% CI [1.04, 1.29], p=0.008) (data not shown). However, in the ordinal logistic regression analysis, the test of parallel lines was significant when type 2 diabetes was placed in the model instead of insulin, which indicated the proportional odds assumption was violated. Thus, BMI and insulin were adjusted for in the regression analysis because they appeared to be more of a possible confounder than type 2 diabetes.

From the 47 original age-matched pairs, 24 discordant pairs were used for the conditional logistic regression of the presence of CAC (Table 4.9). C3 was significantly related to the prevalence of CAC after adjusting for case control status (OR=1.29, 95% CI [1.01, 1.65], p=0.04) (Table 4.10). This association of C3 and CAC was attenuated after adjustment for insulin quartiles (p=0.31) or BMI (p=0.47). There was no evidence of an interaction with case control status and C3 (p=0.39).

## 4.4 DISCUSSION

This is the first study to investigate complement protein C3, CVD risk factors and subclinical CVD in middle-aged women with PCOS and their respective controls. Although not significant, women with PCOS had higher C3 levels when compared to controls in women <45

years old (p=0.76) and in women ≥45 years old (p=0.28). C3 significantly correlated with traditional CVD risk factors and higher circulating C3 levels were associated with the presence of CAC and with increasing CAC. Our results indicate that C3 may be a specific atherosclerotic inflammatory marker in women with PCOS and non-PCOS controls.

This study reflected a trend of higher circulating C3 levels in women with PCOS when compared to controls in both age groups, which was more evident in women ≥45 years old. Similar to our results, *Wu et al.* found that C3 levels were higher, but not significantly different, between premenopausal women with PCOS and controls (2.1 g/L versus 1.8 g/L, p>0.05, respectively).[43] They also showed C3 was positively correlated with insulin, homeostasis model assessment-insulin resistance (HOMA-IR) and triglycerides in women with PCOS, all p<0.05. Other studies have shown higher C3 levels in premenopausal women with PCOS compared to their control counterpart. Oktenli *et al.* showed C3 levels were higher among non-obese women with PCOS compared to age and BMI-matched controls (1.4 g/L versus 1.0 g/L, p<0.001, respectively).[44] Similarly, Yang *et al.* showed women with PCOS had higher C3 levels than controls (1.4 g/L versus 1.1 g/L, p<0.05, respectively), and remained higher among women with PCOS after stratifying by lean and obese participants.[45] In addition to evaluating C3 levels, many studies have shown higher levels of circulating inflammatory markers such as C-reactive protein (CRP)[7,46-48] and cytokines[49,50] in women with PCOS when compared to controls.

Complement levels are not traditionally evaluated in the diagnosis or treatment of PCOS. Complement C3 and C4 are circulating markers of inflammation that are routinely measured in the diagnosis and treatment of SLE. C3 has correlated positively with subclinical CVD measures in women with SLE and is considered a SLE-specific risk factor for CVD. Women with PCOS are also at increased risk of premature cardiovascular disease[51] and are affected by many traditional CVD risk factors[52] that could contribute to inflammation. Recent studies

suggest PCOS is a low-grade inflammatory state[48,53,54] similar to autoimmune diseases such as SLE and atherosclerosis in cardiovascular disease.

Women with PCOS have a high prevalence of obesity and insulin resistance, which are also considered to have an underlying inflammatory process.[55-57] Cytokines from adipose tissue[58] and insulin resistant states[59] can stimulate the synthesis of C3. The liver is the main source of C3, but adipocytes secrete inflammatory cytokines and complement proteins including C3.[58,60,61] These observations suggest that obesity and insulin resistance are sources of cytokines and complement that could exacerbate a chronic low grade inflammatory response. An additional complexity arises with a C3 cleavage product termed acylation-stimulating protein (ASP) that is involved in glucose and triglyceride metabolism in adipose tissue.[62,63]

This present analysis showed that C3 had a strong relationship between insulin and BMI, and all of which had significant associations with CAC. This finding is consistent with studies that showed C3 had the strongest correlations with insulin and various features of insulin resistance.[31] Most women with PCOS are obese and insulin resistant,[6,64] which could be linked by chronic inflammation.[54] Studies have suggested C3 is a stronger inflammatory marker of insulin resistance than CRP in non-PCOS populations[65-67] and in women with PCOS.[45] These present findings also indicate C3 could be a key inflammatory marker of obesity and insulin resistance in women with and without PCOS.

A previous analysis of this study population showed a higher presence of CAC (10+) in women with PCOS,[8] which is supported by studies evaluating CAC in premenopausal women with PCOS and controls.[9,10] In this secondary analysis, circulating inflammatory marker complement C3 was also associated with CAC in these participants. The association of C3 and CAC was partially explained by the combination of insulin and BMI, but remained significant. In fully adjusted models, C3 was significantly related to the presence of CAC and borderline

significant in the association of increasing CAC groups. This suggests C3 may be a specific inflammatory marker of atherosclerosis in women with PCOS and non-PCOS controls.

Significant relationships between C3 and subclinical CVD measures including vascular stiffness, as measured by PWV, and CAC have been confirmed in SLE patients and are consistent with these findings in women with PCOS and controls with respect to CAC. Among women with SLE, high C3 levels (>0.9 g/L) were associated with the prevalence of CAC (OR 4.0, p=0.007) after adjusting for age.[35] Increasing C3 levels were also associated with an increase in PWV[36] and with the highest quartile of PWV (OR 1.02, 95% CI [1.00,1.04], p=0.03) in women with SLE.[37]

Additional epidemiological studies have also suggested that C3 is an inflammatory CVD risk factor comparable to traditional CV risk factors,[30,47] and a strong predictor of both initial[48] and recurrent[49] CVD events. Ajjan *et al.* showed C3 was a better predictor of coronary artery disease than CRP in both men and women, and those with higher C3 levels were three times more likely to develop coronary heart disease (CHD).[32] This result was corroborated by Onat *et al.* who showed the odds of CHD was 3.5 for higher C3 levels,[33] while Szeplaki et al. showed high C3 levels (≥1.8 g/L) predicted future vascular complications among women with existing severe CHD.[68] These previous reports support the present findings that C3 strongly correlated with traditional CVD risk factors and CAC within this PCOS population and their respective controls.

The elevation of circulating C3 in healthy and non-healthy individuals with CVD and the relation to subclinical measures suggests a potential mechanistic involvement of complement in the development of atherosclerosis. Complement proteins could play a role in vascular dysfunction by deteriorating the mechanical integrity of the vascular wall, and over time this could lead to the formation and progression of an atherosclerotic lesion.[37] Atherosclerotic lesions have been found to contain complement proteins including C3.[69-71] Conventional

thought suggests that various complement activating substrates within atherosclerotic lesions activate complement leading to additional inflammation and further lesion progression. Given the evidence, C3 may confer an increased risk of CVD by contributing to vascular stiffness, which normally occurs with age in healthy populations,[72,73] and atherosclerosis.

A limitation to this study was the length of serum sample storage. The samples were stored for an average of 9 years; however, Muscari *et al.* successfully evaluated C3 levels in samples stored for 7 years.[74] Another limitation was that participants were lost to follow up from the original CHARM I visit. However, this analysis included a large population of women with PCOS and controls and there was a wide range of age and BMI when compared to the literature. Another strength was that the sensitivity analysis with a subset of the original age matched pairs showed that C3 was related to the presence of CAC after adjusting for case control status. The cross-sectional design of this study limited the ability to determine causality; however previous prospective studies mentioned previously have shown C3 significantly predicted CVD events.

Despite these limitations, this study remains the first to investigate C3 levels among middle-aged women with PCOS compared to controls and to investigate the association of C3 with CVD risk factors and subclinical CVD. Future studies are needed to confirm these results and could be further investigated by using other markers of subclinical atherosclerosis. Studies have shown levels of C3 significantly decreased after administering an insulin-sensitizing medication for three months in women with PCOS[44] and decreased with weight loss and physical activity in men.[75] It would be interesting to evaluate whether reducing C3 has a beneficial effect on subclinical measures of atherosclerosis.

In conclusion, there is evidence that circulating serum C3 is associated with subclinical cardiovascular disease. Previous reports from this study population showed that PCOS was associated with CAC, but this analysis showed that C3 is also independently associated with

CAC. Our results have important public health implications as this indicates C3 may be a specific inflammatory CVD risk marker in women with and without PCOS. Further investigations of the inflammatory mechanisms behind the progression of atherosclerosis are needed to identify vulnerable subgroups at risk for CVD. Ultimately, this could lead to targeted therapies to reduce inflammation and prevent the progression of atherosclerosis.

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## 4.6 TABLES AND FIGURES FOR CHAPTER FOUR

Table 4.1 Characteristics of cases and controls by age group

| | Age <45 | | | Age ≥45 | | |
|---|---|---|---|---|---|---|
| | Cases (n=55) | Controls (n=39) | P-value | Cases (n=77) | Controls (n=116) | P-value |
| Age (yr) | 41.96±2.09 | 42.00±2.25 | 0.92 | 50.44±3.79 | 51.75±4.21 | **0.03** |
| Complement C3 (mg/dL) | 171.20±37.86 | 168.79±35.11 | 0.76 | 175.12±39.85 | 169.06±36.81 | 0.28 |
| BMI (Kg/m$^2$) | 31.41±7.13 | 29.23±6.61 | 0.14 | 31.53±7.91 | 28.34±5.94 | **0.002** |
| Waist Circumference (cm) | 92.40±16.62 | 87.06±14.07 | 0.11 | 94.08±18.02 | 85.68±14.04 | **<0.0001** |
| Waist-to-Hip Ratio[1] | 0.84±0.09 | 0.80±0.07 | **0.04** | 0.85±0.09 | 0.81±0.08 | **<0.0001** |
| SBP (mm Hg) | 114.26±10.52 | 113.82±12.74 | 0.86 | 120.90±11.38 | 118.41±14.64 | 0.21 |
| DBP (mm Hg) | 74.20±8.48 | 73.97±9.01 | 0.90 | 77.43±8.11 | 75.94±8.15 | 0.22 |
| Insulin (μU/mL)* | 17.90 (10.10, 26.90) | 11.80 (9.00, 19.60) | **0.04‡** | 15.40 (9.60, 24.30) | 10.90 (8.40, 15.38) | **<0.0001‡** |
| Glucose (mg/dL)* | 90.00 (85.23, 97.00) | 89.18 (85.00, 96.19) | 0.94‡ | 93.00 (86.50, 105.00) | 93.00 (87.00, 98.62) | 0.18‡ |
| QUICKI | 0.32±0.03 | 0.33±0.02 | 0.07 | 0.32±0.04 | 0.33±0.03 | **0.001** |
| Triglycerides (mg/dL)* | 104.00 (73.00, 199.00) | 96.00 (79.20, 127.00) | 0.39‡ | 140.00 (86.80, 233.00) | 115.00 (77.93, 154.25) | **0.02‡** |
| Total Cholesterol (mg/dL) | 201.07±38.75 | 194.74±31.91 | 0.40 | 214.43±47.00 | 214.28±35.16 | 0.98 |
| HDLc (mg/dL) | 52.52±13.37 | 52.12±11.23 | 0.88 | 52.00±17.18 | 58.34±14.90 | **0.007** |
| LDLc (mg/dL)[2] | 120.54±35.51 | 119.05±26.77 | 0.83 | 127.90±43.26 | 130.19±33.31 | 0.68 |

Values are mean±SD or *median (Inter-Quartile Range: 25th, 75th percentile); ‡P-value by Mann-Whitney U test, otherwise by unpaired t test; 1) n=38 cases <45, n=76 cases ≥45; 2) n=75 cases age ≥45

Table 4.2 Hormones in cases and controls by age group (excluding women on OC or HRT)

| | Age <45 | | | Age ≥45 | | |
|---|---|---|---|---|---|---|
| | Cases (n=48) | Controls (n=32) | P-value | Cases (n=57) | Controls (n=85) | P-value |
| Age (yr)* | 41.87±2.17 | 41.81±2.41 | 0.91 | 50.44±3.62 | 51.29±3.95 | 0.19 |
| BMI ($Kg/m^2$)* | 31.95±7.33 | 28.50±6.11 | **0.03** | 32.41±7.89 | 28.41±6.24 | **0.001** |
| Total Testosterone (ng/dL)[1] | 25.94 (19.88, 45.39) | 20.03 (19.88, 25.94) | **0.005** | 23.05 (19.88, 46.11) | 23.05 (19.88, 28.82) | 0.39 |
| Sex Hormone Binding Globulin (nmol/mL) | 81.90 (53.43, 130.80) | 129.35 (83.45, 177.05) | **0.02** | 75.10 (54.50, 138.45) | 126.60 (80.00, 189.30) | **0.0005** |
| Luteinizing hormone- (miU/mL)[2] | 7.55 (4.05, 11.88) | 5.00 (2.60, 7.90) | 0.08 | 11.90 (5.95, 21.90) | 24.50 (6.92, 44.00) | **0.0002** |
| FSH (mIU/ml)[2] | 5.45 (4.70, 6.78) | 6.30 (3.90, 10.90) | 0.51 | 14.50 (7.30, 34.30) | 41.10 (12.10, 78.70) | **<0.0001** |
| Progesterone (ng/dL)[1] | 2.45 (1.60, 5.35) | 4.25 (1.53, 41.30) | 0.26 | 1.80 (1.30, 5.00) | 1.50 (1.10, 2.68) | 0.13 |
| Estradiol (pg/mL)[3] | 79.05 (55.55, 133.98) | 76.50 (35.75, 123.85) | 0.38 | 54.20 (35.55, 92.40) | 40.30 (26.60, 90.85) | 0.41 |

Values are median (Inter-Quartile Range: 25th, 75th percentile) or *mean±SD ; P-value by Mann-Whitney U test or t-test; 1) n=84 controls ≥45; 2) n=31 controls <45, n=83 controls ≥45; 3) n=36 cases, 25 controls <45, n=45 cases, 72 controls ≥45

Table 4.3 Selected characteristics by age group

| | Age <45 | | | Age ≥45 | | |
|---|---|---|---|---|---|---|
| | Cases (n=55) n (%) | Controls (n=39) n (%) | P-value | Cases (n=77) n (%) | Controls (n=116) n (%) | P-value |
| High Complement C3 (>180 mg/dL, Quest reference range 90-180 mg/dL) | 25 (45.5) | 14 (35.9) | 0.35 | 32 (42.1) | 39 (33.6) | 0.23 |
| African American Race | 6 (10.9) | 8 (20.5) | 0.20 | 7 (9.1) | 23 (19.8) | **0.04** |
| Current Smoker | 8 (14.5) | 9 (23.1) | 0.29 | 15 (19.5) | 13 (11.2) | 0.11 |
| Current OC user | 6 (10.9) | 5 (12.8) | 1.0* | 4 (5.3) | 5 (4.3) | 0.74* |
| Current HRT user | 1 (1.8) | 2 (5.3) | 0.57* | 16 (20.8) | 26 (22.4) | 0.79 |
| Never been pregnant | 11 (20.0) | 7 (17.9) | 0.80 | 20 (26.0) | 21 (18.1) | 0.19 |
| Postmenopausal (no period in the last 12 months) | 5 (9.1) | 5 (12.8) | 0.74* | 30 (39.0) | 58 (50.0) | 0.13 |
| Obese (BMI ≥30) | 30 (54.5) | 15 (38.5) | 0.12 | 45 (58.4) | 42 (36.2) | **0.002** |
| Hypertension Treated | 9 (16.4) | 3 (7.7) | 0.35* | 23 (29.9) | 24 (20.7) | 0.15 |
| Type 2 Diabetes, Doctor Diagnosed | 5 (9.1) | 1 (2.6) | 0.40* | 12 (21.4) | 3 (4.8) | **0.006** |
| Type 2 Diabetes (doctor diagnosed) or glucose ≥126 (mg/dL) | 5 (9.1) | 2 (5.1) | 0.70* | 13 (16.9) | 4 (3.4) | **0.001** |

Values are number (percent); P-value between cases and controls by chi-square test or *Fisher's Exact test

Table 4.4 Coronary artery calcium by age group

| | Age <45 | | | Age ≥45 | | |
|---|---|---|---|---|---|---|
| | Cases (n=55) n (%) | Controls (n=39) n (%) | P-value | Cases (n=77) n (%) | Controls (n=116) n (%) | P-value |
| CAC Any | 29 (52.7) | 15 (38.5) | 0.20 | 50 (64.9) | 51 (44.0) | **0.004** |
| CAC 10+ | 15 (27.3) | 8 (20.5) | 0.50 | 27 (35.1) | 14 (12.1) | **<0.0001** |
| CAC groups | | | | | | |
| 0 | 26 (47.3) | 24 (61.5) | 0.40 | 27 (35.1) | 65 (56.0) | **<0.0001** |
| 1-10 | 14 (25.5) | 7 (17.9) | | 23 (29.9) | 37 (31.9) | |
| ≥11 | 15 (27.3) | 8 (20.5) | | 27 (35.1) | 14 (12.0) | |

Values are number (percent); CAC expressed as Agatston Score; P-value between cases and controls by chi-square test

Table 4.5 Spearman's correlations with complement C3 by age group

| | Age <45 | | Age ≥45 | |
|---|---|---|---|---|
| | Cases (n=55) | Controls (n=39) | Cases (n=77) | Controls (n=116) |
| Age (yr) | -0.03 (0.83) | 0.24 (0.14) | 0.08 (0.50) | **0.20 (0.03)** |
| BMI (Kg/m$^2$) | **0.59 (<0.0001)** | **0.52 (0.0006)** | **0.69 (<0.0001)** | **0.58 (<0.0001)** |
| Waist Circumference (cm) | **0.60 (<0.0001)** | **0.60 (<0.0001)** | **0.71 (<0.0001)** | **0.64 (<0.0001)** |
| Waist-to-Hip Ratio[1] | **0.46 (0.0005)** | **0.52 (0.0007)** | **0.56 (<0.0001)** | **0.55 (<0.0001)** |
| SBP (mm Hg) | 0.21 (0.12) | 0.20 (0.22) | **0.27 (0.02)** | **0.37 (<0.0001)** |
| DBP (mm Hg) | 0.22 (0.10) | 0.25 (0.13) | 0.21 (0.07) | **0.44 (<0.0001)** |
| Insulin (µU/mL)* | **0.58 (<0.0001)** | **0.63 (<0.0001)** | **0.64 (<0.0001)** | **0.57 (<0.0001)** |
| Glucose (mg/dL)* | **0.32 (0.02)** | 0.006 (0.97) | **0.50 (<0.0001)** | **0.33 (0.0003)** |
| QUICKI | **-0.59 (<0.0001)** | **-0.60 (<0.0001)** | **-0.66 (<0.0001)** | **-0.57 (<0.0001)** |
| Triglycerides (mg/dL)* | **0.55 (<0.0001)** | **0.36 (0.03)** | **0.50 (<0.0001)** | **0.34 (0.0002)** |
| Total Cholesterol (mg/dL) | **0.52 (<0.0001)** | **0.58 (0.0001)** | **0.25 (0.03)** | 0.16 (0.09) |
| HDLc (mg/dL) | **-0.30 (0.03)** | -0.10 (0.56) | **-0.38 (0.001)** | -0.18 (0.05) |
| LDLc (mg/dL)[2] | **0.36 (0.008)** | **0.50 (0.001)** | 0.21 (0.08) | 0.10 (0.28) |

Values are Spearman's rho (p-value)

Table 4.6 Spearman's correlations of complement C3 and hormones among cases and controls by age groups (excluding women on OC or HRT)

| | Age <45 | | Age ≥45 | |
|---|---|---|---|---|
| | Cases (n=55) | Controls (n=39) | Cases (n=77) | Controls (n=116) |
| Total Testosterone (ng/dL)[1] | -0.09 (0.55) | -0.07 (0.71) | 0.03 (0.85) | 0.04 (0.72) |
| Sex Hormone Binding Globulin (nmol/mL) | **-0.46 (0.001)** | **-0.42 (0.02)** | **-0.48 (0.0002)** | **-0.50 (<0.0001)** |
| Luteinizing Hormone (miU/mL)[2] | -0.06 (0.67) | 0.25 (0.18) | -0.20 (0.13) | 0.17 (0.12) |
| Follicle Stimulating Hormone (mIU/ml)[2] | **0.29 (0.04)** | 0.27 (0.14) | -0.07 (0.59) | 0.08 (0.47) |
| Progesterone (ng/dL)[1] | -0.13 (0.38) | -0.09 (0.63) | -0.13 (0.35) | -0.09 (0.42) |
| Estradiol (pg/mL)[3] | -0.31 (0.07) | -0.31 (0.13) | -0.10 (0.51) | **-0.25 (0.04)** |

Values are Spearman's rho (p-value); 1) n=84 controls ≥45; 2) n=31 controls <45, n=83 controls ≥45; 3) n=36 cases, 25 controls <45, n=45 cases, 72 controls ≥45

Table 4.7 Logistic regression analysis of the presence of CAC (Agatston score ≤0 vs. >0)

| | | OR | 95% CI | p-value |
|---|---|---|---|---|
| Model 1 | Age (yrs) | 1.05 | 1.00, 1.09 | **0.042** |
| | PCOS (vs. Control) | 2.26 | 1.39, 3.70 | **0.001** |
| Model 2a | Age (yrs) | 1.04 | 0.99, 1.09 | 0.127 |
| | PCOS (vs. Control) | 2.31 | 1.32, 4.06 | **0.003** |
| | Complement C3 (per 10 mg/dL) | 1.39 | 1.27, 1.52 | **<0.0001** |
| Model 2b | Age (yrs) | 1.06 | 1.00, 1.11 | **0.038** |
| | PCOS (vs. Control) | 1.82 | 1.01, 3.28 | **0.046** |
| | Complement C3 (per 10 mg/dL) | 1.26 | 1.14, 1.39 | **<0.0001** |
| | Insulin Quartiles | 1.86 | 1.37, 2.53 | **<0.0001** |
| Model 3 | Age (yrs) | 1.10 | 1.04, 1.16 | **0.002** |
| | PCOS (vs. Control) | 1.79 | 0.95, 3.37 | 0.072 |
| | Complement C3 (per 10 mg/dL) | 1.14 | 1.03, 1.27 | **0.011** |
| | BMI ($kg/m^2$) | 1.25 | 1.17, 1.35 | **<0.0001** |
| Model 4 | Age (yrs) | 1.10 | 1.04, 1.17 | **0.001** |
| | PCOS (vs. Control) | 1.58 | 0.81, 3.05 | 0.176 |
| | Complement C3 (per 10 mg/dL) | 1.11 | 1.00, 1.24 | 0.057 |
| | BMI ($kg/m^2$) | 1.23 | 1.14, 1.33 | **<0.0001** |
| | Insulin Quartiles | 1.29 | 0.90, 1.85 | 0.167 |
| Model 5 | Age (yrs) | 1.10 | 1.04, 1.17 | **0.001** |
| | PCOS (vs. Control) | 1.79 | 0.91, 3.53 | 0.092 |
| | Complement C3 (per 10 mg/dL) | 1.12 | 1.00, 1.25 | **0.049** |
| | BMI ($kg/m^2$) | 1.22 | 1.13, 1.32 | **<0.0001** |
| | Insulin Quartiles | 1.30 | 0.90, 1.87 | 0.166 |
| | African American (vs. White) | 2.83 | 1.09, 7.34 | **0.033** |

Table 4.8 Ordinal regression analysis of CAC groups (Agatston score 0, 1-10, and ≥11)

| | | OR | 95% CI | p-value |
|---|---|---|---|---|
| Model 1 | Age (yrs) | 1.03 | 1.00, 1.06 | 0.058 |
| | PCOS (vs. Control) | 1.91 | 1.35, 2.71 | **0.0003** |
| | Complement C3 (per 10 mg/dL) | 1.21 | 1.15, 1.27 | **<0.0001** |
| Model 2a | Age (yrs) | 1.04 | 1.00, 1.07 | 0.027 |
| | PCOS (vs. Control) | 1.58 | 1.10, 2.26 | **0.013** |
| | Complement C3 (per 10 mg/dL) | 1.14 | 1.08, 1.20 | **<0.0001** |
| | Insulin Quartiles | 1.54 | 1.27, 1.89 | **<0.0001** |
| Model 2b | Age (yrs) | 1.04 | 1.00, 1.07 | **0.034** |
| | PCOS (vs. Control) | 1.57 | 1.09, 2.26 | **0.015** |
| | Complement C3 (per 10 mg/dL) | 1.09 | 1.03, 1.15 | **0.003** |
| | BMI ($kg/m^2$) | 1.14 | 1.11, 1.18 | **<0.0001** |
| Model 3 | Age (yrs) | 1.04 | 1.01, 1.07 | **0.017** |
| | PCOS (vs. Control) | 1.46 | 1.00, 2.12 | **0.048** |
| | Complement C3 (per 10 mg/dL) | 1.06 | 1.00, 1.12 | 0.071 |
| | BMI ($kg/m^2$) | 1.14 | 1.10, 1.18 | **<0.0001** |
| | Insulin Quartiles | 1.31 | 1.06, 1.62 | **0.012** |
| Model 4 | Age (yrs) | 1.04 | 1.01, 1.07 | **0.015** |
| | PCOS (vs. Control) | 1.68 | 1.13, 2.48 | **0.01** |
| | Complement C3 (per 10 mg/dL) | 1.06 | 0.99, 1.12 | 0.082 |
| | BMI ($kg/m^2$) | 1.13 | 1.09, 1.17 | **<0.0001** |
| | Insulin Quartiles | 1.29 | 1.05, 1.60 | **0.017** |
| | African American Race (vs. White) | 1.73 | 1.09, 2.75 | **0.021** |

Table 4.9 Frequency of CAC (Agatston Score 0 vs. > 0) in the matched pairs of women with PCOS and controls*

| **Pairs (N=47)** | | Controls | |
|---|---|---|---|
| | | No CAC (0) | CAC (1) |
| Cases | No CAC (0) | 12 | 8 |
| | CAC (1) | 16 | 11 |

*47 original age-matched pairs (n=94 women), 24 discordant pairs (n=48 women)

Table 4.10 Conditional logistic regression analysis of CAC (Agatston Score 0 vs. >0) among women with PCOS and controls (N=94)

| | | B (S.E.) | p-value | OR (95% CI) |
|---|---|---|---|---|
| Model 1 | Case (vs. Control) | 0.35 (0.22) | 0.11 | 2.00 (0.86, 4.67) |
| Model 2 | Case (vs. Control) | 0.38 (0.26) | 0.14 | 2.14 (0.78, 5.83) |
| | Complement C3 (per 10 mg/dL) | 0.25 (0.12) | **0.04** | 1.29 (1.01, 1.65) |
| Model 3 | Case (vs. Control) | 0.16 (0.29) | 0.31 | 1.38 (0.44, 4.35) |
| | Complement C3 (per 10 mg/dL) | 0.11 (0.01) | 0.45 | 1.12 (0.84, 1.48) |
| | Insulin Quartiles | 0.83 (0.42) | 0.05 | 2.30 (1.00, 5.28) |
| Model 4 | Case (vs. Control) | 0.22 (0.30) | 0.47 | 1.54 (0.48, 4.94) |
| | Complement C3 (per 10 mg/dL) | 0.004 (0.18) | 0.98 | 1.00 (0.71, 1.43) |
| | BMI ($kg/m^2$) | 0.21 (0.13) | 0.09 | 1.24 (0.97, 1.59) |

47 original age-matched pairs, 24 discordant pairs (n=48 women); CI, 95% Wald Confidence Intervals

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# 5.0 TRAJECTORY ANALYSIS OF ABSOLUTE CHANGE IN BRACHIAL LUMEN DIAMETER AMONG WOMEN WITH POLYCYSTIC OVARY SYNDROME AND CONTROLS

## 5.1 INTRODUCTION

Flow-mediated dilation (FMD) is a widely used non-invasive measure of endothelial function. Endothelial dysfunction indicates early vascular injury and is a functional measure of subclinical atherosclerosis.[1,2] Endothelial dysfunction has been shown in animal models of atherogenesis,[3] in individuals with atherosclerosis[4,5] and in women with polycystic ovary syndrome (PCOS).[6-9] PCOS is the most common reproductive endocrine disorder that affects 6-10% of the women in the United States.[10,11] Women with PCOS experience acne, excessive hair, weight gain and irregular periods. Women with PCOS have an increase in cardiovascular disease (CVD) risk factors,[7,12-15] and subclinical atherosclerosis as measured by coronary artery calcification[16-19] and carotid artery intima-media thickness when compared to controls.[2,20-26] Studies have shown women with PCOS have lower FMD compared to controls,[6-9] but some also have shown no differences.[27,28] These contradicting results could be due to low sample sizes and the variability of the endothelial function test.

The endothelial function test is difficult to measure and is prone to multiple sources of error or variance. Endothelial function is assessed with B-mode ultrasound to measure changes in brachial artery diameter in response to an increase in sheer stress after transient ischemia. FMD is expressed as the percent change in lumen diameter (LD) from baseline in response to

increased flow after transient ischemia, and is calculated by taking the maximum LD divided by the baseline LD times 100.[4] Lower FMD in response to an increased blood flow indicates endothelial dysfunction. FMD is around 5% to 15% in most individuals, but lower or missing in individuals with cardiovascular disease.[29]

Endothelial function is affected by many physiological and participant factors, which increases the variability of FMD and decreases the power of a study to detect associations with FMD. Lower endothelial dysfunction has been associated with age, BMI,[30] hypertension,[31,32] inflammation,[33,34] metabolic factors such as dyslipidemia and insulin resistance,[1,35,36] and psychosocial factors including stress,[37] Bortner Type A behavior, Spielberger trait anger and Beck depression scores.[38] In addition to these CVD risk factors, FMD varies with race,[39] point in the menstrual cycle,[40] with estrogens and menopausal status,[41,42] and with alcohol[43] and smoking.[44] Endothelial function may also vary by the time of day, but this has not been consistently shown.[45,46] Medications such as ACE inhibitors, statins, antioxidants, insulin sensitizers and L-arginine improve endothelial function.[47,48] Endothelial function is also positively related to adiponectin, vitamin B and folic acid,[49] exercise,[47] tea,[50] and cocoa.[51] Furthermore, factors that decrease brachial LD would intrinsically cause higher FMD levels because smaller vessels are able to dilate, or compensate for an increase in blood flow and sheer stress, more than larger vessels.[4] Also, the rate of the stimulus stress, which is believed to be an important determinant of the vascular response, varies across participants and could add to the variability of FMD.[52]

Technical factors during the endothelial function assessment and differences in reading scans also contribute to the variability of FMD since the protocol for endothelial function is not standardized. Variations in the protocol include inflating blood pressure cuff up to different pressures, occluding the vessel from 4 to 5 minutes and obtaining images at specific times. Bots *et al.* showed that the location and duration of occlusion increases FMD and could add to

the variability of FMD.[53] FMD was higher in the upper arm versus lower arm, and the longer duration of occlusion was associated with a larger FMD. However, the investigators showed that technical factors have minimal contribution to the variability of FMD compared to traditional CVD risk factors.

Taken together, these sources of variability make it difficult to interpret the data. There are no guidelines for data management such as how to handle negative values and outliers. To try to decrease the variability of FMD, investigators could take a new approach by using group-based modeling to analyze the results from the endothelial function assessment.

Semi-parametric group-based trajectory modeling, also called latent class growth modeling, is commonly used to model development and behavior in criminology and psychology.[54] This method assumes there are subgroups in a population that follow similar trajectories. SAS Proc Traj models patterns of change in an outcome across multiple time points and identifies subgroups in the population.[55,56] The advantage of this method is that the groups are not defined a priori, which differs from standard growth modeling.

This method could identify and summarize subgroups of different patterns of absolute change in lumen diameter over the time course of the endothelial function test. The analysis could identify participants who have a lower response and evaluate the associations between covariates and group membership. This would improve the understanding of the endothelial test by removing the variability of FMD due to calculating the percent change in lumen diameter. It would also detect patterns of change, rather than an individual's FMD value.

For this analysis, it is assumed participants can be grouped into different trajectories based on the change in LD after transient ischemia during the endothelial function test. The aim of this paper was to determine whether there are distinct trajectories of absolute change in LD after transient ischemia among women with PCOS and controls, and whether PCOS status or other characteristics are associated with group trajectories.

## 5.2 METHODS

### 5.2.1 Study Population

This analysis is based from the Cardiovascular Health and Risk Measurement Study (CHARM) of women with PCOS and non-PCOS controls. The investigators, Talbott and Sutton-Tyrrell, described the methodology and recruitment of the original study previously.[57] Briefly, they identified women aged 19-55 diagnosed with PCOS between 1970 and 1993 through medical records in the Division of Reproductive Endocrinology at Magee-Women's Hospital (Pittsburgh, PA), and recruited women through private practices and the local chapter of the PCOSA support group. The University of Pittsburgh institutional review board approved the protocols, and all participants gave consent before enrolling. Investigators obtained a clinical diagnosis of PCOS at baseline, defined by the NIH as a history of anovulation and either (1) clinical evidence of androgen excess (hirsutism) or an elevated testosterone level (0.2 nmol/L) or (2) a luteinizing hormone/follicle-stimulating hormone ratio (LH/FSH) greater than 2.0. Investigators matched PCOS cases to controls by age (±5 years) and race. The neighborhood controls came from voter's registration tapes and Cole's Cross Reference Directory of Households from 1993.

The present study was based from the second follow-up of CHARM that occurred during 1997 to 1999 where 335 women 30-60 years old were contacted to assess measures of subclinical cardiovascular disease. This present analysis included 128 women with PCOS and 148 controls that underwent the endothelial function ultrasound assessment during the 1997 to 1999 follow-up visit (Figure 5.1).

### 5.2.2 Covariates

The clinical assessments for participant characteristics have been previously described.[20,57,58] Briefly, investigators collected height, weight, BMI, waist and hip circumference (cm), waist to hip ratio, blood pressure, and serum concentrations of hormones, total cholesterol, LDLc, HDLc, triglycerides, fasting glucose and insulin, C-reactive protein and hormones that included sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone, progesterone and estradiol. Participants completed a questionnaire on medical, surgical, menstrual and reproductive history, medication use, lifestyle, and family history of PCOS. For this analysis, the quantitative insulin sensitivity check index (QUICKI) was calculated (QUICKI= 1/[log fasting insulin concentration (μU/mL) + log glucose concentration (mg/dL)]).[59] QUICKI is a measure of insulin sensitivity where lower values indicate insulin resistance.

### 5.2.3 Endothelial Function Assessment

Flow-mediated dilation was measured by Toshiba SSA-270A and Hewlett Packard 5500 duplex scanners. Technicians placed a cuff on the right forearm and an ultrasound probe two inches above the antecubital fossa. The procedure began after the patients rested for ten minutes. The sonographer recorded a one minute baseline digital and then inflated the cuff to 50 mmHg above the systolic blood pressure for four minutes to induce hyperemia. The sonographer recorded digital images post deflation every thirty seconds for two minutes. The sonographer measured the brachial LD from digital images timed to end diastole of the cardiac cycle. Three measurements of the LD for each time point were averaged.

### 5.2.4 Statistical Methods

**Data preparation**

To prepare the data, variables were created that represented the 30 second time variable and the corresponding absolute change in LD (time 0 and rchg0, time 1 and rchg1, etc.). The absolute change in LD at time zero was set to zero for all participants. The censored normal distribution model was in SAS Proc Traj was used, which was developed by Nagin and colleagues to model group trajectories. The single outcome was absolute change in LD at the 30 second time points.

**Model Selection and Diagnostics**

The optimal number of groups was determined for up to 5 groups using second order polynomial models by evaluating model fits using the BIC criteria and the estimated log Bayes factor (Table 5.1). For this data, a three-group trajectory model was identified as the optimal number of groups. Some group sizes were less than five percent for models exceeding three groups. Once the three group model was determined to be the optimal model, the cubic term was added to each group. Non-significant terms were removed from each group and models were evaluated using the BIC criteria.

To check models, trajectories were graphed with the 95% confidence intervals, and the average posterior probabilities of membership in each group (AvePP) and the odds of correct classification $(OCC)^2$ were calculated (Table 5.2).[54] The OCC was calculated by: $OCC_j=[(AvePP_j/1\text{-}AvePP_j)/(\pi_j/1\text{-}\pi_j)]$; where $\pi_j$ is the estimated probability of group membership. More accurate group assignments correspond to a larger OCC. Adequate models should have an AvePP of >0.70 and an OCC of >5.

**Characteristics by Group**

Posterior probabilities were used to classify participants into groups. Descriptive statistics were computed for categorical and continuous variables by groups. Differences in levels of characteristics across groups were evaluated using ANOVA for normally distributed variables, the Welsh test for normally distributed variables with unequal variances across groups, the Kruskal-Wallis Test for non-normally distributed variables, and the Chi-square or Fisher's exact tests for categorical variables. Women using hormone replacement therapy or oral contraceptives were excluded when analyzing hormone levels so the results were not influenced by hormone use.

Group membership was modeled using multinomial logistic regression analyses to identify possible characteristics associated with trajectories. The multinomial model evaluated differences in variables between non-dilators and enhanced dilators compared to the dilators as the reference. Group two was selected as the reference group because it contained the largest number of women and was hypothesized to represent the endothelial response for a general population. Non-normal variables were transformed and the log of insulin and glucose were divided by the standard deviation for ease of interpreting model parameters. The variables were modeled adjusting for baseline lumen diameter, a major component of the response of the change in lumen diameter. Variables with a p-value $<0.20$ were assessed in multivariable models using forward stepwise selection. All first order interactions with PCOS status were evaluated. Boxplots were created for the significant variables to illustrate differences by group. SAS (release 9.2; SAS, Cary, NC) was used for the trajectory modeling and all other analyses were done with PASW (version 18; IBM SPSS Inc., Chicago, IL).

## 5.3 RESULTS

The analysis identified three distinct patterns of absolute change in LD, which were labeled as enhanced dilators, dilators, non-dilators (Figure 5.2). There were 117 (42.4%) women classified as non-dilators, 123 (44.6%) women classified as dilators and 36 (13%) women classified as enhanced dilators.

When descriptive characteristics were compared across groups, insulin levels were significantly different between non-dilators, dilators and enhanced dilators, p=0.03 (Table 5.3). The other descriptive characteristics were not significantly different across groups. Similarly, the hormone, inflammatory, fibrinolytic and coagulation factors were not different across the groups (Tables 5.4 and 5.5). However, significant differences were seen in endothelial function parameters that included baseline lumen diameter, maximum change in lumen diameter, absolute change in lumen diameter, and FMD (Table 5.6).

The distribution of women with PCOS was not different across groups as there were 42.7% of the non-dilators were women with PCOS, 46.3% of the dilators were women with PCOS, and 58.3% of the enhanced dilators were women with PCOS, p=0.26 (Table 5.7). The other categorical variables were not different by group. When stratified by women with PCOS and controls, the selected factors were also not significantly different across groups (Table 5.8). There were borderline significant differences across groups with respect to insulin and glucose among the controls.

In the multinomial logistic regression analysis, larger baseline lumen diameter was associated with a 2.05 greater likelihood of non-dilators compared to dilators (95% CI [1.09, 3.87], p=0.03) (Table 5.9). After adjusting for PCOS, age and baseline lumen diameter, lower insulin was associated with a greater likelihood of non-dilators compared to dilators (OR 0.70, 95% CI [0.52, 0.93], p=0.02), and higher HDLc was borderline significantly associated with a

greater likelihood of non-dilators compared to dilators (OR=1.02, 95% CI [1.00, 1.04], p=0.05). Boxplots of these variables also illustrated these associations with group membership (Figures 5.3 thru 5.5). There was a significant interaction term with cholesterol quartiles and case control status for the non-dilators compared to dilators (OR 0.55, 95% CI [0.34, 0.90], p=0.02) after adjusting for age, baseline lumen diameter and insulin quartiles. The graph of the predicted probabilities of the non-dilators from the interaction model by case control status showed that as cholesterol quartiles increases, the probability of non-dilators versus dilators decreases for cases and increases for controls (Figure 5.6). This suggests that higher cholesterol is associated with a greater likelihood of being a dilator in cases, but being a non-dilator in controls. There was no evidence of other interactions with case control status. The results of all the descriptive and regression analyses did not change when excluding women on medications for insulin and glucose.

## 5.4 DISCUSSION

This analysis identified three distinct patterns of absolute change in lumen diameter during the time course of the endothelial function test. Most of the women were classified as non-dilators and dilators, 35.2% and 46.1%, respectfully, whereas 13.0% were classified as enhanced dilators. The results are surprising because it suggests 35.2% of the women have evidence of a low endothelial response indicative of endothelial dysfunction. Insulin and the endothelial function parameters were the only variables that were different across groups. The proportion of women with PCOS did not differ between groups. Factors associated with the non-dilators compared to the dilators included higher baseline lumen diameter after adjusting for case control status and age, and lower insulin and higher HDLc after adjusting for case control

status, age and baseline lumen diameter. There was a significant interaction between total cholesterol and PCOS status in which increasing cholesterol quartiles were associated with non-dilators in controls but associated with dilators in cases.

This analysis showed that a larger baseline diameter was associated with non-dilators compared to dilators (3.04±0.41 versus 2.92±0.43, p=0.03, respectively). These findings fall within the range of reported baseline LDs of 2 to 7 mm,[60] and are similar to studies that showed a negative association of FMD with baseline LD.[60-62] As expected, women in the non-dilator group had significantly lower absolute change in lumen diameter compared to dilators (0.08±0.08 versus 0.22±0.06, p<0.0001) and the enhanced dilators had significantly higher absolute change compared to dilators (0.40±0.08 versus 0.22±0.06, p<0.0001). The absolute change in LD has been shown to range from -0.07 to 0.71 mm.[60] In women, the mean absolute change has been shown to be anywhere from 0.43±0.02 mm,[63] 0.27±0.13 mm[62] to 0.139±0.002 mm.[39] Increases in LD are a normal process of arterial remodeling that occurs in response to changes in blood flow to preserve vascular tone and sheer stress.[64] However, larger baseline diameters have less ability to respond to increases in blood flow.[4] A larger baseline LD has been associated with lower endothelial function,[65,66] coronary artery calcium,[67] and coronary artery disease[5,65] and a smaller absolute change has been associated with peripheral arterial disease.[68]

Besides baseline LD, the other strongest determinant of the non-dilators was insulin in which lower insulin levels were associated with non-dilators compared to dilators. Insulin is involved in vasodilation,[69-74] and affects NO synthesis[74] This might seem counterintuitive given that insulin is involved in vasoconstriction,[75] and insulin resistance[6] and diabetes[35,48] are associated with lower FMD. However, insulin resistance blunts the vasodilating signaling of insulin, whereas the vasoconstricting signaling is unchanged.[75] This could explain why lower insulin was associated with non-dilators.

An unexpected finding was that higher HDLc was associated with non-dilators compared to dilators. However, this was borderline significant at p=0.05. Evidence suggests that under certain conditions, the beneficial actions of HDLc are lost and can become a pro-inflammatory factor (piHDLc). Levels of piHDLc have been shown to be higher in inflammatory states, such as in women with systemic lupus erythematosus (SLE),[76] and associated with carotid intima-media thickness in women with SLE.[77] The function of HDLc was independent of HDLc serum levels in these studies, but suggests a potential mechanism for the association of higher HDLc and non-dilators.

There was a significant interaction between cholesterol and PCOS status in which higher cholesterol quartiles were associated with non-dilators among the controls, but associated with dilators among the cases. Studies have shown higher total cholesterol is associated with reduced endothelial function.[78] However, total cholesterol has not been associated with FMD in women with PCOS and controls (spearman correlation r=0.045, p=0.71).[6] A study among women with PCOS showed that the duration of reactive hyperemia (durRH), defined as the time for the blood flow to return to baseline levels after reactive hyperemia, was shorter in women with PCOS versus controls (63.75±13.33 seconds versus 113.18±20.92 seconds, p=0.036).[79] The investigators showed that total cholesterol was not significantly related to the durRH, but the spearman correlations were in opposite directions between cases (r=-0.17, p=0.49) and controls (r=0.14, p=0.68). Thus, the interaction between case control status and group membership could reflect differences between women with PCOS and controls in how cholesterol affects stimulus stress or other mechanisms of the endothelial response after reactive hyperemia.

Underlying explanations for these findings are that these factors could affect important regulators of vascular function such as nitric oxide (NO) function[74] and oxidative stress. In addition, differences in stimulus stress could explain the results. Stimulus stress is a major

determinant of FMD along with baseline LD,[80,81] but not always measured in conjunction with endothelial function. Recently researchers recommend that the dilation should be corrected for the rate of stimulus stress achieved during hyperemia, as this varies across participants and could account for variations in FMD.[80,82] However, correcting for the stimulus stress is under debate because there might not be an association between sheer stress and FMD in certain populations and study designs,[83] or the association may not be linear.[84]

Many published guidelines recommend studies to report baseline LD, absolute change in LD and FMD, area under the curve (AUC) and time to maximum diameter.[56] This would help to interpret the results from the endothelial function assessment and reduce variability across studies. In addition, investigators have tried to decrease the variability and increase sensitivity of the endothelial function data by using various analytic methods. Investigators have used AUC[85] as an alternative to FMD and shown it is more sensitive in detecting endothelial responses over the time course of the measurement than FMD.[60] However, this assumes investigators have continuous accurate measures of LD over the time course. This is not easy to obtain because of the technical precision that it entails. Also, some studies measure LD at specific time points rather than continuously. Other investigators have looked at the time course[86] and used it to classify responders based on the AUC of FMD.[61]

These methods are similar to this trajectory analysis because the participant endothelial responses were classified into groups. This analysis also allowed for the adjustment for baseline LD when analyzing the absolute change, which is not recommended when analyzing FMD because baseline LD is part of the calculation. Another benefit of this analysis was that the absolute change has been shown to be more consistent than FMD with changing baseline LDs.[60]

A limitation of this study was that the LD was measured at 30 second intervals. This might have underestimated the true maximum diameter, but may have a small impact on the

results because the focus was on patterns of change rather than the maximum dilation. Another limitation was that endothelium-independent dilation was not measured so the function of the smooth muscle cells on the endothelial response could not be evaluated. Also, stimulus stress was not measured, which has been suggested to be as important as baseline LD in determining the endothelial response to sheer stress.[80,82] However, this limitation exists in most studies because sheer stress is rarely assessed and adjusting for the stimulus stress is still under debate.

Despite these limitations, this trajectory analysis identified individuals with similar patterns of absolute change in LD and identified factors associated with group membership. It would be important to replicate this method in different populations while taking into account the aforementioned variability in the endothelial response due to baseline LD and stimulus stress. Larger studies may be needed to increase the power of this analysis to identify factors associated with group membership. Future studies could look longitudinally and see if individuals fall into the same trajectory group over time. Studies could also evaluate the effect changes in CVD risk factors have on group membership.

In summary, baseline lumen diameter, insulin and HDLc were identified as important factors in the endothelial response after reactive hyperemia. There was also evidence that the association of total cholesterol and group membership was different between women with PCOS and controls. Clearly much remains to be known about the mechanisms of vasoreactivity. Endothelial function is a complex process; many factors play a role and it varies under different conditions. Thus, multiple methods should be used to analyze and interpret the results from the endothelial function assessment. Applying this analytic method to past and future studies of endothelial function will allow researchers to evaluate the utility of this method as it could be a less variable way to analyze endothelial data compared to current methods. In

conclusion, this analysis showed promise to be an additional tool to understand factors affecting endothelial function and identify individuals with endothelial dysfunction.

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## 5.6 TABLES AND FIGURES FOR CHAPTER FOUR

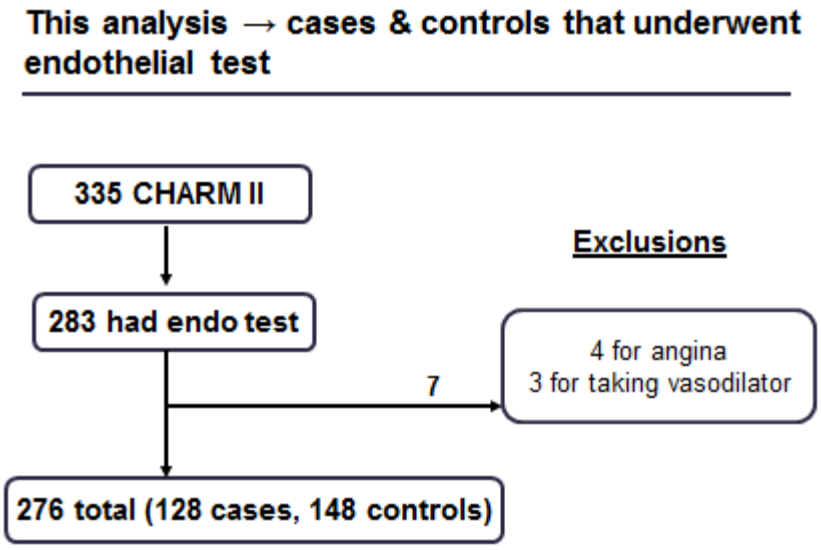


Figure 5.1 Case and control selection

Table 5.1 Model selection based on the log Bayes factor

| No. of Groups | BIC (N=128) | $2(\Delta BIC) \approx 2\log_e(B_{10})$ |
|---|---|---|
| 1 | 1237.47 | |
| 2 | 1586.9 | 698.86 |
| 3 | 1684.06 | 194.32 |
| 4 | 1741.41 | 114.7 |
| 5 | 1759.33 | 35.84 |

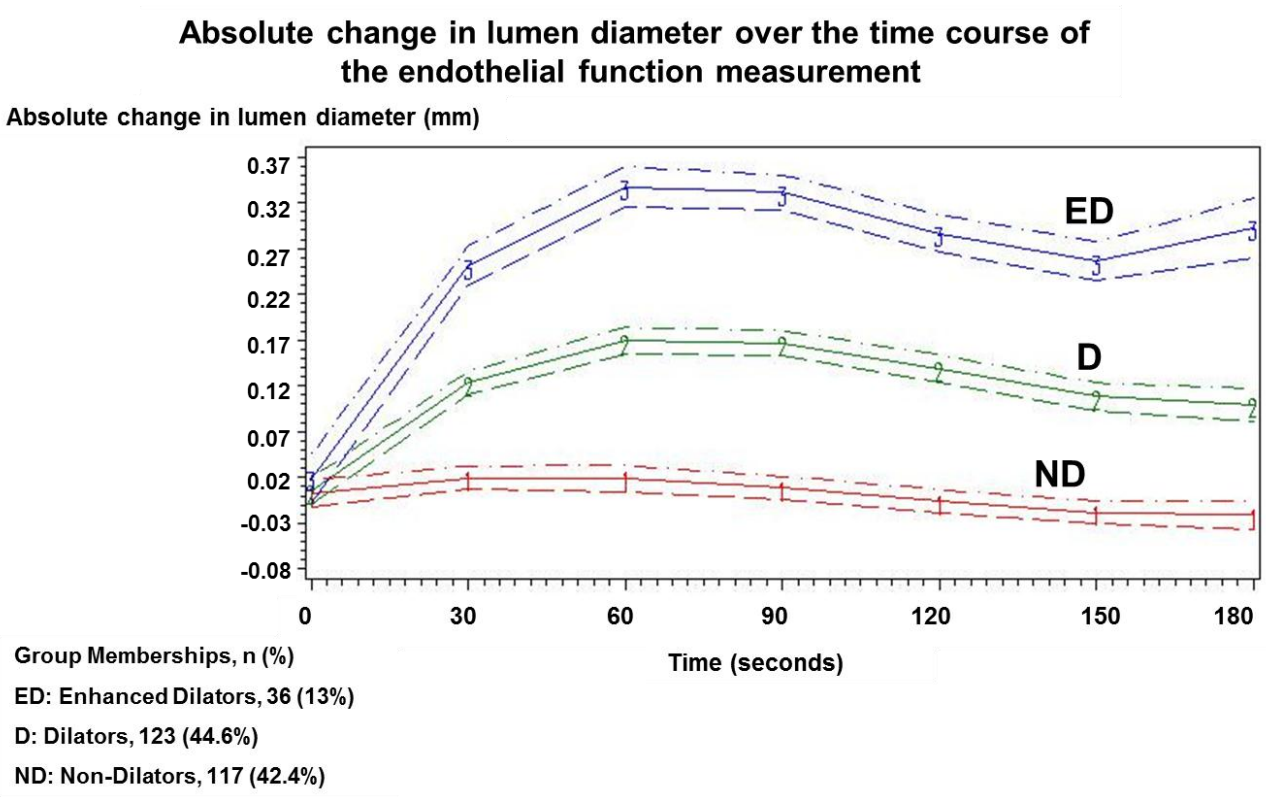


Figure 5.2 Absolute change in lumen diameter after reactive hyperemia

Table 5.2 Diagnostics for the three group model

| Group | AvePP | Estimated Group Membership Probabilities ($\pi_j$) | OCC |
|---|---|---|---|
| Non-Dilators | 0.95 | 0.43 | 25.69 |
| Dilators | 0.93 | 0.44 | 16.99 |
| Enhanced Dilators | 0.98 | 0.14 | 311.09 |

Average Group Probabilities (AvePP); Odds of Correct Classification (OCC)

Table 5.3 Descriptive variables for women with PCOS and controls aged 30-60 from CHARM II endothelial function test by group

| | N | All | n | Non-Dilators | n | Dilators | n | Enhanced Dilators | p-value |
|---|---|---|---|---|---|---|---|---|---|
| Age (yr) | 276 | 42.55±7.02 | 117 | 42.50±7.24 | 123 | 42.72±6.86 | 36 | 42.13±7.03 | 0.90 |
| Weight (lbs.) | 276 | 172.93±44.50 | 117 | 169.77±42.32 | 123 | 175.12±49.15 | 36 | 175.71±33.79 | 0.57ǂ |
| Waist Circumference (cm) | 264 | 87.48±16.49 | 109 | 86.44±16.82 | 120 | 87.71±17.19 | 35 | 89.89±12.68 | 0.55 |
| Waist-to-hip ratio | 263 | 0.80±0.07 | 109 | 0.79±0.08 | 119 | 0.80±0.07 | 35 | 0.81±0.06 | 0.25ǂ |
| BMI ($Kg/m^2$) | 276 | 29.21±7.21 | 117 | 28.54±6.81 | 123 | 29.66±7.88 | 36 | 29.82±5.94 | 0.42 |
| SBP (mm Hg) | 276 | 114.64±14.86 | 117 | 115.57±16.02 | 123 | 114.36±14.65 | 36 | 112.61±11.38 | 0.56 |
| DBP (mm Hg) | 276 | 74.27±9.10 | 117 | 74.75±9.02 | 123 | 73.82±9.10 | 36 | 74.25±9.56 | 0.73 |
| Insulin (μU/mL)* | 275 | 12.90 (9.60, 18.50) | 116 | 12.00 (9.20, 16.73) | 123 | 13.20 (9.70, 20.70) | 36 | 14.95 (11.03, 23.05) | **0.03*** |
| Glucose (mg/dL)* | 272 | 90.05 (83.92, 97.07) | 116 | 91.37 (84.79, 97.73) | 121 | 89.18 (82.16, 97.07) | 35 | 87.42 (82.16, 94.44) | 0.25* |
| QUICKI | 272 | 0.32±0.03 | 116 | 0.33±0.02 | 121 | 0.32±0.03 | 35 | 0.32±0.02 | 0.17 |
| Triglycerides (mg/dL)* | 276 | 110.00 (75.00, 156.00) | 117 | 103.50 (70.25, 141.25) | 123 | 110.00 (77.00, 163.00) | 36 | 137.50 (89.00, 163.00) | 0.15 |
| Total Cholesterol (mg/dL) | 276 | 205.34±35.62 | 117 | 204.28±35.23 | 123 | 205.20±35.63 | 36 | 209.22±37.54 | 0.77 |
| HDLc (mg/dL) | 276 | 56.83±15.20 | 117 | 58.69±15.57 | 123 | 56.18±15.39 | 36 | 53.07±12.64 | 0.12 |
| LDLc (mg/dL) | 273 | 123.05±32.61 | 114 | 121.39±30.20 | 123 | 123.07±32.63 | 36 | 128.21±39.62 | 0.55 |
| Leptin (ng/mL)* | 238 | 19.10 (11.60, 31.13) | 93 | 18.60 (10.05, 32.15) | 113 | 19.00 (11.70, 32.30) | 32 | 19.65 (13.30, 28.03) | 0.91* |

Values are the mean±SD or *median (Inter-Quartile Range: 25th, 75th percentile); p-value by ANOVA or ǂWelsh test or *Kruskal-Wallis Test; excluding women on insulin meds (n=7) or excluding women on hypoglycemic meds (n=9) did not change results

Table 5.4 Hormone variables by group (excluding women currently using OCs or HRTs)

| | N | All | n | Non-Dilators | n | Dilators | n | Enhanced Dilators | p-value |
|---|---|---|---|---|---|---|---|---|---|
| Age (yr) | 146 | 41.49±6.19 | 58 | 41.46±6.37 | 67 | 41.60±5.85 | 21 | 41.26±7.01 | 0.98 |
| BMI ($Kg/m^2$) | 146 | 30.85±7.57 | 58 | 29.98±7.77 | 67 | 31.22±7.94 | 21 | 32.03±5.61 | 0.49 |
| Sex Hormone Binding Globulin (nmol) | 146 | 175.81±91.42 | 58 | 195.19±94.62 | 67 | 166.02±87.02 | 21 | 153.50±90.27 | 0.10 |
| Free Testosterone (nmol/L)* | 144 | 1.57 (0.93, 2.46) | 58 | 1.39 (0.79, 2.45) | 66 | 1.46 (1.02, 2.44) | 20 | 1.95 (1.46, 2.95) | 0.14* |
| Total Testosterone (ng/dL)* | 146 | 36.52 (24.07, 56.66) | 58 | 35.18 (23.78, 54.50) | 67 | 37.27 (22.34, 59.69) | 21 | 37.21 (29.28, 49.43) | 0.94 |
| Luteinizing hormone (miU/mL)* | 123 | 5.27 (2.29, 10.52) | 45 | 5.75 (2.76, 9.28) | 58 | 5.37 (2.63, 10.77) | 20 | 2.94 (1.68, 11.45) | 0.56 |
| Estradiol (pg/mL)* | 117 | 70.60 (46.20, 106.95) | 45 | 73.90 (41.45, 110.75) | 52 | 71.10 (41.68, 112.18) | 20 | 64.90 (51.13, 96.38) | 0.38 |
| Estrone (pg/mL)* | 117 | 45.70 (26.50, 60.50) | 45 | 45.30 (25.95, 60.75) | 52 | 45.80 (28.60, 60.58) | 20 | 43.30 (22.33, 62.70) | 0.47 |
| Free estradiol index (estradiol/SHBG)* | 96 | 0.47 (0.26, 0.83) | 37 | 0.65 (0.22, 0.85) | 42 | 0.55 (0.30, 0.85) | 17 | 0.43 (0.24, 0.99) | 0.96 |

Values are the mean±SD or *median(Inter-Quartile Range: 25th, 75th percentile); p-value by ANOVA or *Kruskal-Wallis Test

Table 5.5 Inflammatory, fibrinolytic and coagulation factors by group

| | N | All | n | Non-Dilators | n | Dilators | n | Enhanced Dilators | p-value |
|---|---|---|---|---|---|---|---|---|---|
| PAI-1 (ng/mL) | 270 | 18.91 (9.95, 36.00) | 114 | 17.62 (10.15, 33.69) | 121 | 21.00 (9.00, 38.95) | 35 | 19.39 (9.00, 31.00) | 0.71 |
| CRP (mg/dL) | 272 | 1.75 (0.97, 3.34) | 115 | 1.64 (0.90, 3.27) | 122 | 1.65 (0.98, 3.70) | 35 | 2.16 (1.13, 2.85) | 0.73 |
| Factor VIIc (%) | 270 | 125.50 (110.00, 149.25) | 114 | 124.00 (105.50, 149.00) | 121 | 127.00 (116.00, 150.50) | 35 | 125.00 (112.00, 159.00) | 0.39 |
| D_Dimer (ng/mL) | 272 | 91.04 (61.31, 144.87) | 115 | 91.67 (60.24, 150.08) | 122 | 98.16 (60.80, 136.69) | 35 | 85.37 (69.58, 145.23) | 0.99 |
| Fragment 1.2 (nM) | 272 | 1.05 (0.88, 1.32) | 115 | 1.07 (0.89, 1.22) | 122 | 1.02 (0.83, 1.36) | 35 | 1.07 (0.92, 1.40) | 0.72 |

Values are the median (Inter-Quartile Range: 25th, 75th percentile), p-value by Kruskal-Wallis Test

Table 5.6 Endothelial function parameters by group

| | N | All | n | Non-Dilators | n | Dilators | n | Enhanced Dilators | p-value |
|---|---|---|---|---|---|---|---|---|---|
| Baseline Lumen Diameter (mm) | 276 | 2.98±0.43 | 117 | 3.04±0.41 | 123 | 2.92±0.43 | 36 | 3.06±0.48 | **0.05** |
| Maximum Change in Lumen Diameter (mm) | 276 | 3.17±0.45 | 117 | 3.12±0.41 | 123 | 3.14±0.44 | 36 | 3.46±0.47 | **0.0001** |
| Absolute Change in Lumen Diameter (mm) | 276 | 0.19±0.13 | 117 | 0.08±0.08 | 123 | 0.22±0.06 | 36 | 0.40±0.08 | **<0.0001** |
| FMD (%) | 276 | 6.41±4.56 | 117 | 2.77±2.80 | 123 | 7.80±2.41 | 36 | 13.50±3.76 | **<0.0001**‡ |

Values are the mean±SD, p-value by ANOVA or ‡Welch test

Table 5.7 Selected categorical factors by group

| | All N (%) | Non-Dilators n (%) | Dilators n (%) | Enhanced Dilators n (%) | p-value |
|---|---|---|---|---|---|
| PCOS | 128 (46.4) | 50 (42.7) | 57 (46.3) | 21 (58.3) | 0.26 |
| African American Race | 41 (14.9) | 20 (17.1) | 16 (13.0) | 5 (13.9) | 0.66 |
| Age ≥45 years old | 96 (34.8) | 45 (38.5) | 38 (30.9) | 13 (36.1) | 0.46 |
| Education-Highest grade completed | | | | | |
| 10-12 | 68 (24.6) | 30 (25.6) | 29 (23.6) | 9 (25.0) | 0.93 |
| 13-16 | 150 (54.3) | 60 (51.3) | 70 (56.9) | 20 (55.6) | |
| 17 | 58 (21.0) | 27 (23.1) | 24 (19.5) | 7 (19.4) | |
| Current Smoker | 50 (18.1) | 18 (15.4) | 22 (17.9) | 10 (27.8) | 0.24 |
| OC user | 39 (14.1) | 17 (14.5) | 15 (12.2) | 7 (19.4) | 0.54 |
| HRT user | 37 (13.4) | 16 (13.7) | 18 (14.6) | 3 (8.3) | 0.67* |
| Hysterectomy | 19 (6.9) | 9 (7.7) | 7 (5.7) | 3 (8.3) | 0.76* |
| Never been pregnant | 73 (26.4) | 31 (26.5) | 32 (26.0) | 10 (27.8) | 0.98 |
| Postmenopausal (no period in last 12 months) | 57 (20.7) | 27 (23.1) | 25 (20.3) | 5 (13.9) | 0.49 |
| Obesity | | | | | |
| Normal and Overweight (<30) | 164 (59.4) | 72 (61.5) | 72 (58.5) | 20 (55.6) | 0.79 |
| BMI Class 1, 2, 3 (≥30) | 112 (40.6) | 45 (38.5) | 51 (41.5) | 16 (44.4) | |
| High blood pressure doctor diagnosed | 34 (12.3) | 18 (15.4) | 14 (11.4) | 2 (5.6) | 0.31* |
| Type 2 Diabetes doctor diagnosed, or glucose ≥126 mg/dL | 20 (7.2) | 9 (7.7) | 10 (8.1) | 1 (2.8) | 0.65* |
| Hypertriglyceridemia ≥150 mg/dL[1] | 78 (28.3) | 28 (24.1) | 35 (28.5) | 15 (41.7) | 0.13 |

Values are number (percent within group); p-value by Pearson Chi-Square or *Fisher's Exact Tests; NA= Not available; 1) n=116 non-dilators

Table 5.8 Selected factors in women with PCOS and controls by group

| | Women with PCOS | | | | Controls | | | |
|---|---|---|---|---|---|---|---|---|
| | Group 1 (n=50) n (%) | Group 2 (n=57) n (%) | Group 3 (n=21) n (%) | p-value | Group 1 (n=67) n (%) | Group 2 (n=66) n (%) | Group 3 (n=15) n (%) | p-value |
| Age (yrs) | 40.77±6.92 | 42.75±7.30 | 39.98±6.50 | 0.19 | 43.79±7.26 | 42.68±6.52 | 45.13±6.83 | 0.40 |
| BMI (Kg/m$^2$) | 30.25±7.70 | 30.94±8.59 | 31.39±6.27 | 0.83 | 27.27±5.80 | 28.56±7.09 | 27.63±4.82 | 0.50 |
| Insulin (µU/mL)[1] | 14.05 (9.65, 22.55) | 16.20 (10.10, 21.90) | 14.70 (10.40, 25.20) | 0.59 | 11.20 (9.18, 14.25) | 11.60 (9.35, 17.28) | 15.40 (12.30, 18.20) | 0.05 |
| Glucose (mg/dL)[2] | 89.62 (84.57, 95.97) | 91.81 (83.92, 99.70) | 87.42 (81.28, 94.44) | 0.31 | 91.81 (85.45, 98.17) | 87.42 (82.16, 92.90) | 90.05 (82.16, 101.24) | 0.06 |
| QUICKI | 0.32±0.03 | 0.32±0.03 | 0.32±0.02 | 0.61 | 0.33±0.02 | 0.33±0.03 | 0.32±0.02 | 0.14 |
| African American Race | 7 (14.0) | 9 (15.8) | 3 (14.3) | 1.0* | 13 (19.4) | 7 (10.6) | 2 (13.3) | 0.39* |
| Age ≥45 years old | 15 (30.0) | 17 (29.8) | 6 (28.6) | 0.99 | 30 (44.8) | 21 (31.8) | 7 (46.7) | 0.26 |
| Current Smoker | 8 (16.0) | 9 (15.8) | 6 (28.6) | 0.39* | 10 (14.9) | 13 (19.7) | 4 (26.7) | 0.43* |
| OC user | 10 (20.0) | 6 (10.5) | 4 (19.0) | 0.36* | 7 (10.4) | 9 (13.6) | 3 (20.0) | 0.52* |
| HRT user | 7 (14.0) | 9 (15.8) | 1 (4.8) | 0.48* | 9 (13.4) | 9 (13.6) | 2 (13.3) | 1.0* |
| Hysterectomy | 4 (8.0) | 3 (5.3) | 1 (4.8) | 0.89* | 5 (7.5) | 4 (6.1) | 2 (13.3) | 0.59* |
| Never been pregnant | 19 (38.0) | 16 (28.1) | 7 (33.3) | 0.55 | 12 (17.9) | 16 (24.2) | 3 (20.0) | 0.62* |
| Postmenopausal (not had at least 1 period last 12 months) | 7 (14.0) | 11 (19.3) | 1 (4.8) | 0.28* | 20 (29.9) | 14 (21.2) | 4 (26.7) | 0.52* |
| Obesity (BMI ≥30) | 26 (52.0) | 27 (47.4) | 12 (57.1) | 0.73 | 19 (28.4) | 24 (36.4) | 4 (26.7) | 0.58* |
| High Blood Pressure (doctor diagnosed) | 11 (22.0) | 12 (21.1) | 1 (4.8) | 0.20* | 7 (10.4) | 2 (3.0) | 1 (6.7) | 0.22* |
| NIDDM (doctor diagnosed) | 6 (12.0) | 8 (14.0) | 0 (0) | 0.21* | 0 (0) | 1 (1.5) | 0 (0) | 0.55* |
| NIDDM or Glucose ≥126 mg/dL | 8 (16.0) | 8 (14.0) | 1 (4.8) | 0.45* | 1 (1.5) | 2 (3.1) | 0 (0) | 0.72* |

Values are number (percent within group); p-value by Pearson Chi-Square or *Fisher's exact test; NA= Not available; excluding women on insulin meds (n=7) or women on hypoglycemic meds (n=9) did not change results; 1) controls group 1 n=66; 2) PCOS group 2 n=55, controls group 1 n=66 and controls group 3 n=14

Table 5.9 Multinomial logistic regression analysis using the dilator group as the reference

| Model | Group | | Odds Ratio (95% CI) | p-value |
|---|---|---|---|---|
| 1 | Non-Dilators | PCOS (vs. Control) | 0.80 (0.48, 1.35) | 0.41 |
| | | Age (years) | 1.00 (0.96, 1.04) | 0.91 |
| | | Baseline Lumen Diameter (mm) | 2.05 (1.09, 3.87) | **0.03** |
| | Enhanced Dilators | PCOS (vs. Control) | 1.50 (0.70, 3.23) | 0.30 |
| | | Age (years) | 1.00 (0.94, 1.05) | 0.89 |
| | | Baseline Lumen Diameter (mm) | 2.10 (0.87, 5.05) | 0.10 |
| 2 | Non-Dilators | PCOS (vs. Control) | 0.92 (0.54, 1.58) | 0.76 |
| | | Age (years) | 0.99 (0.96, 1.03) | 0.72 |
| | | Baseline Lumen Diameter (mm) | 2.51 (1.29, 4.89) | **0.007** |
| | | Insulin (per standard deviation of the log) | 0.70 (0.52, 0.93) | **0.02** |
| | Enhanced Dilators | PCOS (vs. Control) | 1.48 (0.68, 3.23) | 0.33 |
| | | Age (years) | 1.00 (0.95, 1.05) | 0.93 |
| | | Baseline Lumen Diameter (mm) | 2.10 (0.84, 5.27) | 0.11 |
| | | Insulin (per standard deviation of the log) | 1.04 (0.71, 1.51) | 0.86 |
| 3 | Non-Dilators | PCOS (vs. Control) | 0.84 (0.49, 1.42) | 0.50 |
| | | Age (years) | 0.99 (0.96, 1.03) | 0.71 |
| | | Baseline Lumen Diameter (mm) | 2.44 (1.25, 4.75) | **0.009** |
| | | HDLc (mg/dL) | 1.02 (1.00, 1.04) | 0.05 |
| | Enhanced Dilators | PCOS (vs. Control) | 1.48 (0.69, 3.19) | 0.32 |
| | | Age (years) | 1.00 (0.95, 1.06) | 0.96 |
| | | Baseline Lumen Diameter (mm) | 1.97 (0.78, 5.03) | 0.15 |
| | | HDLc (mg/dL) | 0.99 (0.97, 1.02) | 0.61 |
| 4 | Non-Dilators | PCOS (vs. Control) | 4.00 (1.08, 14.91) | **0.04** |
| | | Age (years) | 0.99 (0.96, 1.04) | 0.78 |
| | | Baseline Lumen Diameter (mm) | 2.70 (1.37, 5.32) | **0.004** |
| | | Insulin (per standard deviation of the log) | 0.72 (0.54, 0.98) | **0.04** |
| | | Cholesterol Quartiles | 1.23 (0.90, 1.69) | 0.20 |
| | | PCOS*Cholesterol Quartiles | 0.55 (0.34, 0.90) | **0.02** |
| | Enhanced Dilators | PCOS (vs. Control) | 6.79 (0.97, 47.63) | 0.05 |
| | | Age (years) | 1.00 (0.94, 1.06) | 0.91 |
| | | Baseline Lumen Diameter (mm) | 2.25 (0.89, 5.66) | 0.09 |
| | | Insulin (per standard deviation of the log) | 1.07 (0.72, 1.58) | 0.74 |
| | | Cholesterol Quartiles | 1.37 (0.83, 2.27) | 0.22 |
| | | PCOS*Cholesterol Quartiles | 0.55 (0.27, 1.10) | 0.09 |

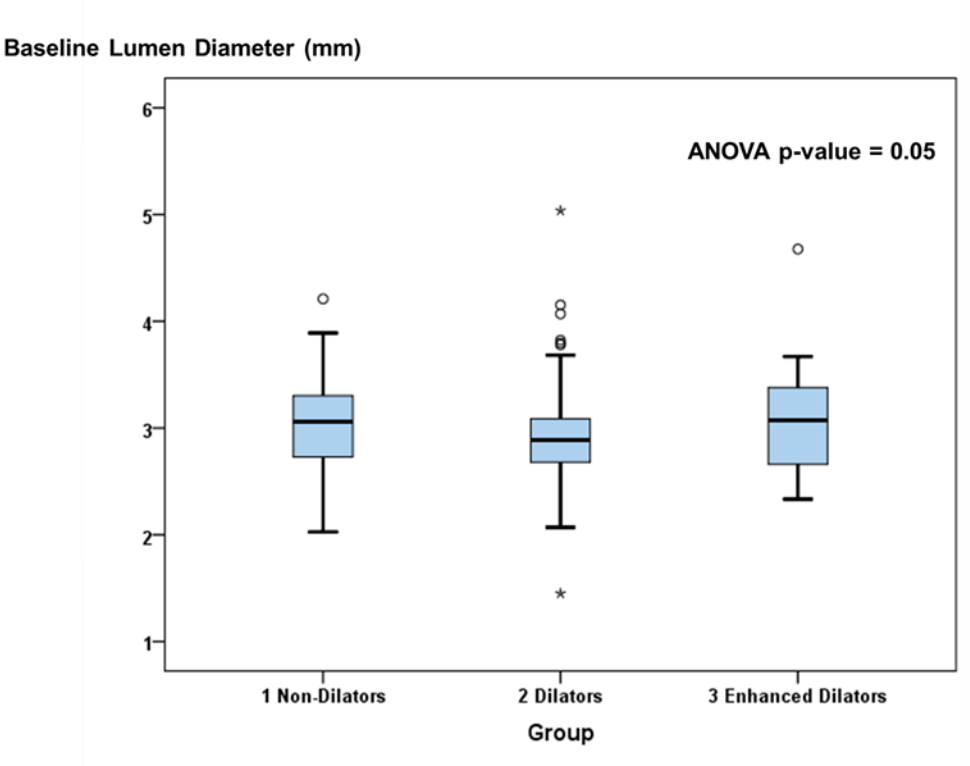


Figure 5.3 Boxplot of baseline brachial lumen diameter by group

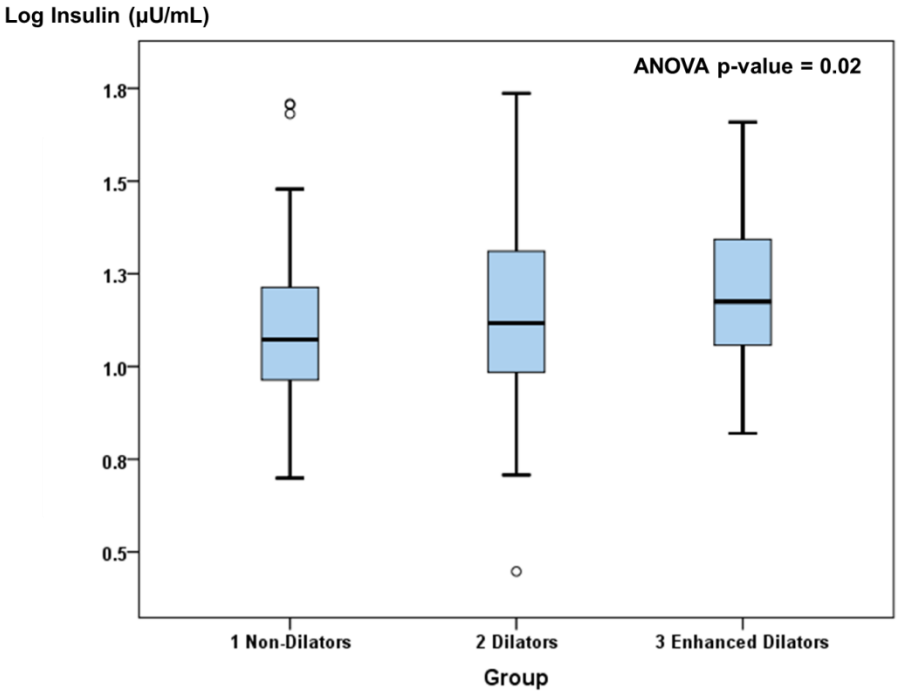


Figure 5.4 Boxplot of insulin by group

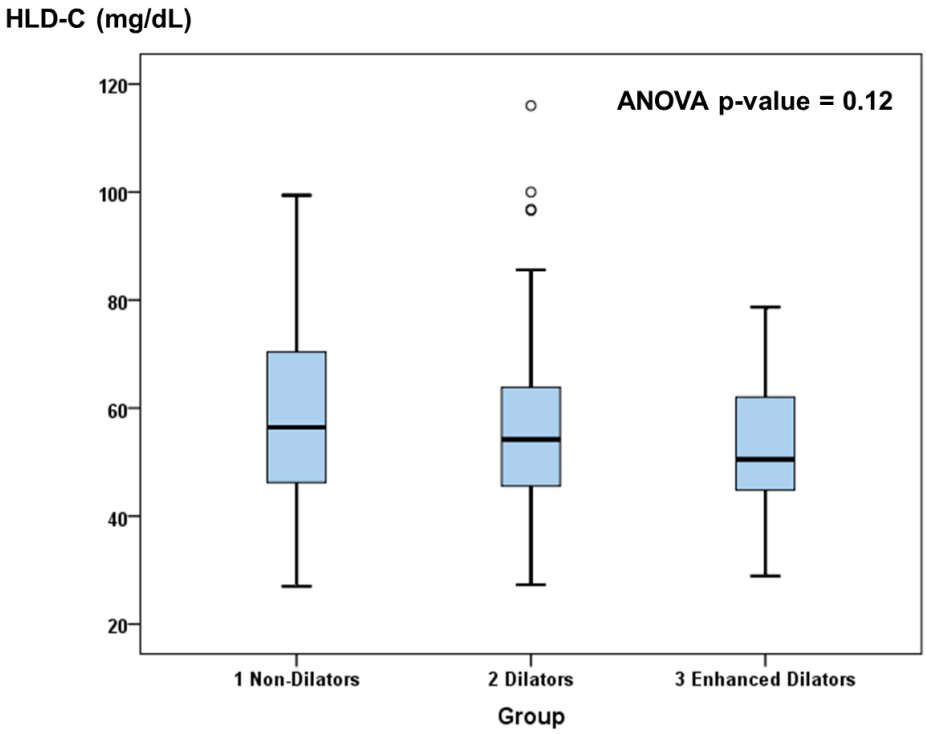


Figure 5.5 Boxplot of HDL-c by group

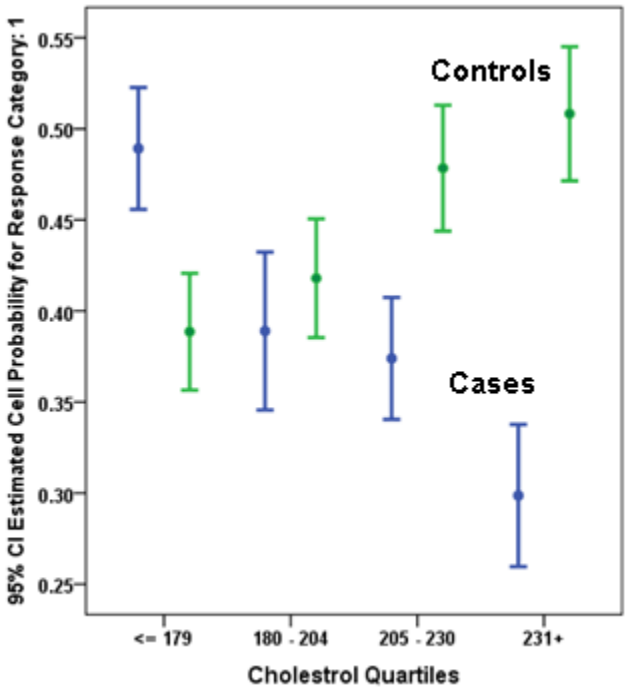


Figure 5.6 Graph of the predicted probabilities of non-dilators compared to dilators from the multinomial logistic regression model with the case control and cholesterol interaction

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# 6.0 GENERAL DISCUSSION

## 6.1 SUMMARY OF FINDINGS

Below is a summary of this dissertation on markers for subclinical cardiovascular disease in women with PCOS and controls. Each chapter evaluated a different measure of subclinical atherosclerosis that included CIMT, CAC and endothelial function.

Chapter three summarized and evaluated the literature on CIMT in women with PCOS compared to controls. The first meta-analysis on subclinical atherosclerosis in women with PCOS indicated women with PCOS had greater CIMT when compared to controls. The summary estimate of the mean difference in CIMT between women with PCOS and controls is comparable to a seven year progression in CIMT. There was significant heterogeneity across studies that may be due to that fact that PCOS is a common complex heterogeneous syndrome. This analysis also revealed that the pattern of CIMT estimates were more constant across studies with the highest quality assessment of CIMT. This highlights the importance using standardized ultrasound protocols and reporting detailed methods as it may explain part of the inconsistencies of the literature.

Chapter four included the first study of the association between complement protein C3, CVD risk factors and subclinical CVD in middle-aged women with PCOS and their respective controls. Although not significant, women with PCOS had higher C3 levels when compared to controls, which was more evident in women ≥45 years old. C3 significantly correlated with

traditional CVD risk factors regardless of age group or case control status. Higher circulating C3 levels were associated with the presence of CAC and with increasing CAC groups after adjusting for case control status and age, and either insulin or BMI. In the fully adjusted model with the aforementioned factors and African American race, C3 was significantly associated with the presence of CAC. The association between C3 and CAC was attenuated when adjusting for insulin and BMI, but was expected since adiposity and insulin resistance are sources of complement and other pro-inflammatory factors. The results indicated that serum C3 is associated with subclinical cardiovascular disease and may be a specific atherosclerotic inflammatory marker in women with and without PCOS.

In chapter five, a novel trajectory analysis was used to analyze the endothelial function data from CHARM. The trajectory analysis identified three distinct patterns of absolute change in lumen diameter during the endothelial function test that were labeled as non-dilators, dilators and enhanced dilators. Most of the women were classified as non-dilators and dilators. Baseline lumen diameter, insulin and HDLc were associated with group membership, and an interactive effect between PCOS status and total cholesterol on group membership was detected. Clearly much remains to be known about the mechanisms of endothelial function. Endothelial function is complex and many factors play a role, which varies under different conditions. Thus, multiple methods should be used to analyze and interpret the results from the endothelial function assessment. This analysis showed promise to be an additional tool to understand factors affecting endothelial function and identify individuals with endothelial dysfunction.

## 6.2 PUBLIC HEALTH SIGNIFICANCE

PCOS is a common reproductive endocrine disorder with short and long term complications for women. Young women might be concerned with cosmetic or reproductive symptoms of PCOS such as hirsutism, acne and infertility, but not with the risk for developing atherosclerosis as they age. The results of this dissertation have important public health implications to guide researchers in developing effective ways to understand the progression of atherosclerosis and to assess CVD risk in women with PCOS. This research is needed to provide a clear consistent message to inform researchers, clinicians and more importantly women with PCOS.

Definitive studies on the increase in CVD events are lacking but many studies show women with PCOS have more CVD risk factors and subclinical atherosclerosis when compared to controls. However, these studies are inconsistent so future meta-analyses could be used to evaluate and summarize the current literature on CVD risk in PCOS. This would provide consistent evidence on the risk of CVD in women with PCOS.

PCOS is considered to be a state of low grade inflammation, but inflammation is not routinely measured in women with PCOS. Complement protein C3 might show promise to be an inflammatory risk marker for CVD in women with and without PCOS. The relation of circulating C3 with subclinical measures in PCOS and non-PCOS individuals suggests a potential mechanistic involvement of complement in the development of atherosclerosis. Further classification of the mechanisms of inflammation on the progression of atherosclerosis is needed to identify vulnerable subgroups at risk for CVD. Ultimately, this could lead to

targeted therapies to reduce inflammation and prevent the progression of atherosclerosis in high risk individuals.

Flow-mediated dilation (FMD) is a widely used measure of subclinical atherosclerosis, but has a lot of variability that makes it hard to interpret the results from the endothelial function assessment. Trajectory analysis could be used to identify subgroups with a low endothelial response and identify important factors that influence endothelial function. Applying this analytic method to past and future studies of flow-mediated dilation would evaluate the utility of this method as it could be a more robust way to analyze endothelial data compared to current methods.

In the absence of definitive studies on the risk of CVD events, PCOS is accompanied by CVD risk factors that place these women at an increased risk of atherosclerosis. The results of this dissertation enforce recommendations for screening and monitoring CVD risk factors in women with PCOS as endorsed by the Androgen Excess PCOS Society, but not yet part of standard care. The results also have implications that can be incorporated into CVD risk assessment and standard of care for PCOS. This is of public health significance as young women with PCOS may not understand the long term effects of PCOS until they are older. Thus, researchers and clinicians need to send consistent messages to young women to prevent the development of CVD risk factors and CVD.

## 6.3 FUTURE RESEARCH

This dissertation showed that various markers of subclinical atherosclerosis could be used to evaluate the risk of CVD in specific populations. It would be important to continue

evaluating the participants from CHARM for subclinical CVD and CVD events. In the meantime, researchers can focus on understanding the mechanisms of CVD in women with PCOS.

The association of C3 with other markers of subclinical atherosclerosis, which include CIMT and FMD, will be investigated in CHARM. However, other studies are needed to confirm the findings that C3 may be an inflammatory risk marker for CVD in women with PCOS and controls. Studies could also determine if lowering C3 has a beneficial effect on subclinical CVD.

Endothelial function is complex and future research is needed to identify factors that affect the endothelial response after reactive hyperemia. It will be important to use multiple methods to analyze data from the endothelial function test. The novel trajectory analysis should be evaluated in different populations to assess the utility of this method. Future studies could look longitudinally and see if individuals are classified into the same trajectory group over time. Studies could also evaluate whether changes in CVD risk factors affect group membership.

In summary, the findings of this dissertation have implications that can be incorporated into CVD risk assessment and standard of care for PCOS. Future research is needed to reduce inconsistencies in the literature by carefully summarizing and evaluating the risk of CVD in women with PCOS. Investigations are also needed to evaluate the utility of novel markers of atherosclerosis and evaluate new methods to analyze endothelial function in women with PCOS. This will move the field of research forward, allowing investigators to understand the complexity of PCOS and the role of CVD risk factors in the progression of CVD in women with PCOS.

## APPENDIX: SYSTEMATIC LITERATURE REVIEW OF FLOW-MEDIATED DILATION AMONG WOMEN WITH PCOS AND CONTROLS

Table A.1 Summary of studies of flow-mediated dilation (FMD) in women with polycystic ovary syndrome and controls

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Mather, 2000 | Age | NIH | Patients | 17 cases | 32.7±1.9[a] | 31.9±2.5[a] | 3.06±0.13 (p=0.42) | 8.7±0.8[a] | 0.53 | Did not adjust for BMI (p<0.05). Small sample size. Did not mention hormone use. Measured post-deflation diameter only at one minute. |
| | | | | 19 controls | 32.4±1.4[a] | 23.3±0.8[a] | 2.93±0.09 | 9.0±0.7[a] | | |
| Talbott, 2001 | Age | NIH | Community | 95 cases | 42.6±6.0 | NA | 3.09±0.5 (p<0.01) | 7.33±5.71 | 0.05 | Analysis limited to Caucasian women over 30 years old. No description on FMD protocol. Baseline lumen diameter was larger among cases (p<0.01). Did not give details about menopausal status or hormone use. |
| | | | | 116 controls | 43.7±6.2 | | 2.88±0.37 | 7.15±4.17 | | |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls
[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

Table A.1 Continued

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Orio, 2004 | Age and BMI | NIH | NA | 30 cases | 22.2±2.5 | 22.4±2.1 | 3.24±0.3 (p<0.5) | 14.3±1.9 | <0.05 | Small sample size. Baseline artery diameter was larger among cases versus controls (p<0.05). No details on control selection. |
| | | | | 30 controls | 22.6±2.3 | 22.1±1.8 | 2.96±0.4 | 18.1±2.0 | | |
| Tarkun, 2004 | Age and BMI | Rotterdam | Doctors and nurses | 37 cases | 23.45±4.3 | 23.85±3.26 | 3.36±0.32 (p=6.8) | 9.93±2.95 | 0.002 | Small sample size. |
| | | | | 25 controls | 24.4±4.07 | 22.9±2.97 | 3.26±0.33 | 14.6±5.15 | | |
| Diamanti-Kandarakis, 2005, Clinical Trial | BMI | NIH | Doctors and nurses | 20 cases | 24.95±1.11[a] | 28.37±1.59[a] | 3.15±0.06 (p=0.48) | 3.24±0.71[a] | <0.0001 | Did not adjust for age (p=0.046). Small sample size. Included smokers. |
| | | | | 20 controls | 26±0.90[a] | 26.59±1.3[a] | 3.24±0.10 | 8.81±1.07[a] | | |
| Kravariti, 2005 | Age and BMI | Rotterdam | NA | 62 cases | 22.69±4.01 | 27.59±5.39 | 3.12±0.39 (p>0.05) | 4.13±2.72 | <0.001 | Small sample size. Measured post-deflation diameter only at one minute. No details on control selection. |
| | | | | 23 lean | 21.83±3.66 | 22.13±1.75 | 2.95±0.34 | 4.60±2.63 | <0.0005 | |
| | | | | 21 over-weight | 21.70±3.70 | 27.67±1.42 | 3.23±0.34 | 4.28±2.79 | | |
| | | | | 18 obese | 24.94±4.08 | 34.49±2.69 | 3.20±0.43 | 3.35±2.74 | | |
| | | | | 17 controls | 24.77±5.66 | 24.96±4.26 | 3.09±0.47 | 9.09±3.99 | | |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls

[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

Table A.1 Continued

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Meyer, 2005 | Age and BMI | NIH | Community | 100 cases<br>20 controls | 32.7±1.8[a]<br>33.2±2.3[a] | 37.3±2.43[a]<br>36.7±1.28[a] | NA | 9.76±0.4[a]<br>13.3±0.9[a] | <0.05 | Had fewer cases than controls. Did not report baseline lumen diameter. Measured post-deflation diameter only at one minute. |
| Alexandraki, 2006 | Age and BMI | NIH | Doctors and medical students | 27 cases<br>27 controls | 25.41±0.80[a]<br>27.33±0.83[a] | 27.42±1.112[a]<br>25.05±1.19[a] | 3.05±0.06[a] (p=0.27)<br>3.17±0.09[a] | 3.84±0.74[a]<br>9.83±0.97[a] | <0.001 | Did not specify the definition of FMD. Studied women with insulin resistance and PCOS; excluded other cardiovascular risk factors. Small sample size. Did not mention smoking status. |
| Brinkworth, 2006 | Weight | Rotterdam | NA | 12 cases<br>10 controls | 31.9±1.8[a]<br>37.2±1.7[a] | 36.2±1.7[a]<br>34.4±1.5[a] | NA | 6.1±1.2[a]<br>5.6±1.0[a] | 0.77 | Did not adjust for age (p=0.05). Did not mention hormone use. Did not report baseline artery diameter. No details on control selection. |
| Carmina, 2006 | Age and weight | NIH | NA | 50 cases<br>50 controls | 25.2±1[a]<br>25.1±0.7[a] | 28.7±0.8[a]<br>28.5±0.5[a] | NA | 15± 0.6[a]<br>18.2± 0.8[a] | <0.05 | Did not take into account hormone use or smoking status. Did not report baseline artery diameter. No details on control selection. |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls
[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

Table A.1 Continued

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Diamanti-Kandarakis, 2006 | Age and BMI | NIH | Doctors and medical students | 25 cases | 25.64±0.86[a] | 29.08±1.43[a] | 3.11±0.29[a] (p=0.24) | 3.47±0.75[a] | <0.001 | Small sample size. |
| | | | | 25 controls | 27.52±1.02[a] | 26.22±1.16[a] | 3.25±0.47[a] | 9.26±0.98[a] | | |
| Sorensen, 2006 | Age and BMI | NIH & Rotterdam | NA | 14 cases | 33.4±3.56 | 25.1±3.75 | NA | 2.49±4.7 | 0.0003 | Small sample size. Did not report baseline artery diameter. Measured post-deflation diameter only at one minute. No details on control selection. |
| | | | | 13 controls | 32.7±5.74 | 24.7±3.90 | | 11.5±5.7 | | |
| Beckman, 2007 | Age and BMI | NIH | News-paper ads and diabetes center | 10 cases | 31±6 | 30±5 | NA | 9.9±0.7[a] | >0.05 | Small sample size. Did not mention hormone use. Did not report baseline artery diameter. Measured post-deflation diameter only at one minute. |
| | | | | 6 lipo-dystrophic women | 47±13 | 27±3 | | 7.7±1.2[a] | >0.05 | |
| | | | | 12 type 2 diabetic women | 56±14 | 31±7 | | 3.4±1.3[a] | 0.02 | |
| | | | | 19 controls | 41±11 | 26±7 | | 7.3±1.1[a] | | |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls
[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

Table A.1 Continued

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Lowenstein, 2007, Clinical Trial | BMI | Rotterdam | Medical staff and colleagues | 31 cases<br>33 controls | 24.3±6.8<br>33.4±11 | 24.2±5.3<br>22.4±4.6 | NA | 1.48±0.32<br>2.00±0.51 | 0.001 | Did not adjust for age (p=0.001). Did not measure insulin resistance. Used the Endo-PAT to measure FMD. Small sample size. Did not mention hormone use. Did not report baseline artery diameter. |
| Battaglia, 2008 | Age and BMI | Rotterdam | NA | 28 cases<br>17 PCO<br>15 controls | 25.2±4.0<br>24.9±3.6<br>26.5±4.4 | 25.2±4.5<br>26.6±6.1<br>25.9±5.1 | NA (p>0.05) | 6 (15 secs), 3 (2 min)[b]<br>10 (15 secs), 4.5 (2 min)[b]<br>11.5 (15 secs), 10.5 (2 min)[b] | <0.05 | Listed baseline artery diameter and FMD in a bar graph and not a table with standard deviations. Small sample size. No details on control selection. |
| Cascella, 2008 | Age and BMI | Rotterdam | NA | 200 cases<br>100 controls | 24.6±3.2<br>24.0±2.8 | 28.5±2.8<br>28.8±2.7 | NA | 13.7±2.3<br>17.8±2.2 | <0.001 | Did not assess smoking status. Did not report baseline artery diameter. No details on control selection. |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls
[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

Table A.1 Continued

| First Author, Year | Control for Age and/or BMI | PCOS Diagnostic Criteria | Control Population | Participants | Age (mean±SD) | BMI (mean± SD) | Baseline Brachial Lumen Diameter (mm), p-value | FMD Endothelial Function % (mean±SD) | P-Value | Limitations |
|---|---|---|---|---|---|---|---|---|---|---|
| Arikan, 2009 | Age and BMI | Rotterdam | Staff and medical students | 39 cases | 22.82±5.53 | 21.48±6.50 | 3.54±0.37 (p>0.05) | 24.88±9.63 | >0.05 | Did not assess hormone use. Small sample size. |
| | | | | 30 controls | 24.64±4.22 | 20.90±6.04 | 3.81±0.66 | 22.35±9.40 | | |
| Luque-Ranurez, 2009 | Age and BMI | Rotterdam | NA | 40 cases | 26±6 | 29.4±6.3 | | | 0.229 | Small sample sizes. Separated data by obesity status. Included smokers. No details on control selection. |
| | | | | Obese (16) | 26±7 | 35.8±3.9 | 0.34±0.06[c] (p=0.065) | 10.3±9.6 | | |
| | | | | non-0bese (24) | 23±5 | 25.1±3.3 | 0.35±0.04[c] | 7.1±10.7 | | |
| | | | | 20 controls | 27±7 | 28.2±6.9 | | | | |
| | | | | Obese (8) | 29±6 | 35.5±3.2 | 0.39±0.04[c] | 5.3±5.4 | | |
| | | | | Non-obese (12) | 26±8 | 23.3±3.2 | 0.36±0.04[c] | 5.6±9.3 | | |
| Soares G, 2009 | Age and BMI | Rotterdam | Basic health clinic | 40 cases | 24.5±3.8 | 22.7±3.3 | 3.00±0.35 (p=0.14) | 8.1±3.6 | 0.80 | Measured post-deflation diameter only at one minute. |
| | | | | 50 controls | 24.5±5.1 | 23.1±3.2 | 3.10±0.34 | 8.36±3.5 | | |

*Values are±SD; FMD, Flow-Mediated Dilation; NA, Not Addressed; P values are cases versus controls
[a]Data expressed as Standard Error (SE), [b]FMD at 15 seconds and 2 minutes, estimated from bar graph, [c]Baseline lumen diameter in cm

**Systematic Literature Review Methods for Flow-Mediated Dilation in Women with Polycystic Ovary Syndrome and Controls**

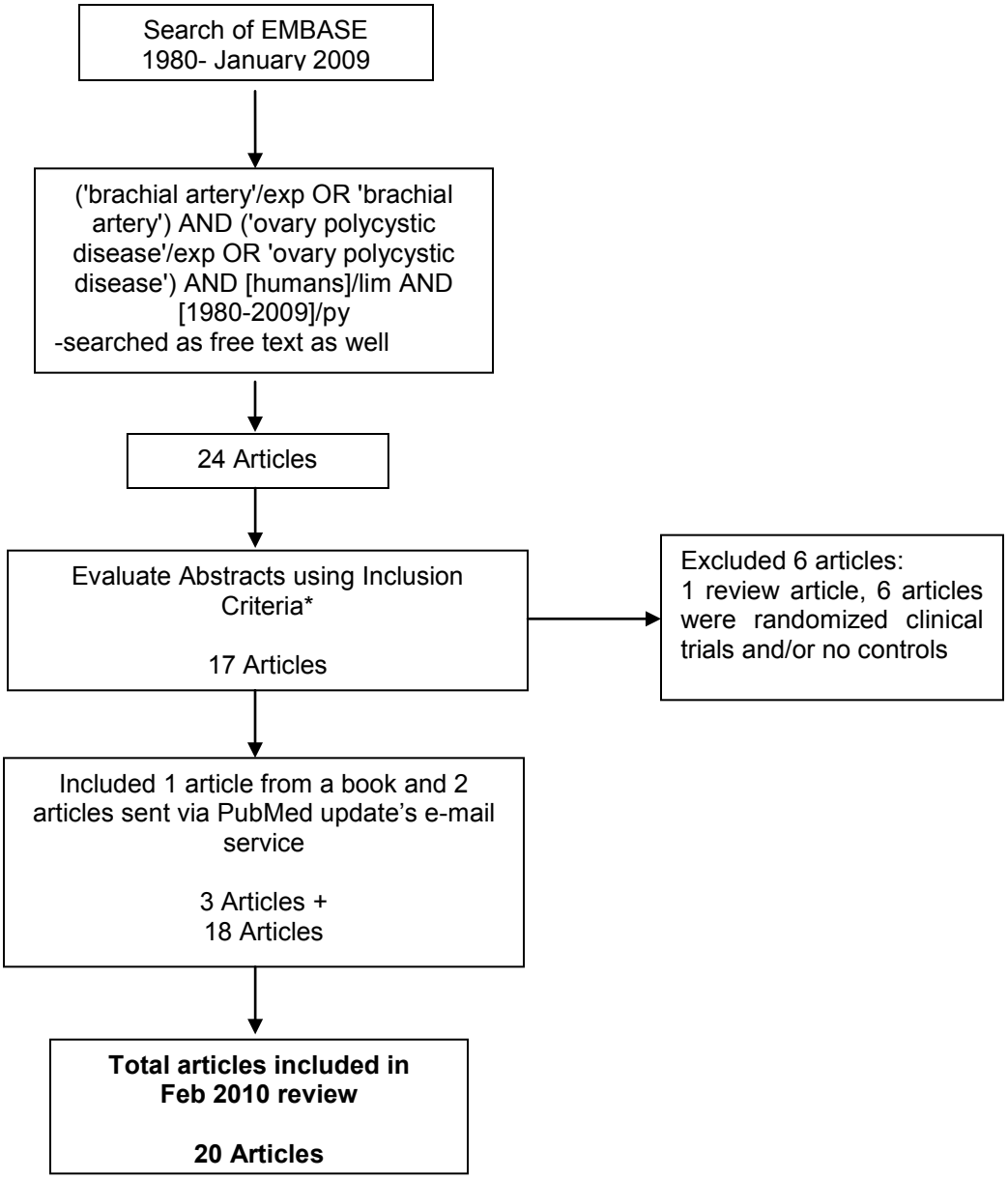


Figure A.1 Diagram of search strategy used to identify articles for review of flow-mediated dilation (FMD) in women with Polycystic Ovary Syndrome (PCOS)*

*Inclusion criteria: primary article with a study population with women with PCOS and controls, and had a measure of FMD

Note: A PubMed search (Brachial Artery/physiopathology"[MeSH Terms] AND ("Polycystic Ovary Syndrome"[MAJR]) did not identify new papers.

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