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Pharmacogenomics

The role of variants regulating metformin transport and action in women with polycystic ovary syndrome

Aims: Variants in genes encoding metformin transport proteins and the *ATM* gene are associated with metformin response. We hypothesized that these gene variants contribute to variable metformin treatment response in polycystic ovary syndrome. **Materials & methods:** The discovery cohort (n = 38) was studied in an open-label study. Results were replicated in two additional cohorts (n = 26 and n = 131). Response was assessed after 3–6 months of treatment with metformin extended-release 1500–2000 mg/day. **Results:** The rs683369 variant was associated with less weight loss in the discovery cohort (p = 0.003), but these results were not replicated (p = 0.8). There were no differences in glucose parameters, testosterone levels or ovulatory frequency as a function of genotype. **Conclusion:** Variants in organic ion transporters do not explain the variable metformin response in polycystic ovary syndrome.

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Insulin resistance has been recognized as important in the pathogenesis of polycystic ovary syndrome (PCOS) in approximately 65% of patients [1,2]. Metformin is widely used to treat women with PCOS based on the concept that it improves insulin resistance. However, we and others have demonstrated that metformin does not improve insulin resistance [3–5]. Rather, it improves fasting glucose levels and glucose-mediated glucose disposal (glucose effectiveness) in women with PCOS [5–10]. Furthermore, metformin lowers testosterone levels and increases ovulatory rates in a subset of women [5] independent of insulin parameters [5]. Thus, the mechanism of metformin action in PCOS remains unclear and identifying the subset of patients who will benefit from metformin remains a challenge.

DNA variants in genes encoding organic ion transporters that are responsible for metformin transport may alter metformin pharmacokinetics [11–15] and could provide insight into the variability of the clinical response to metformin treatment. Metformin is transported into hepatocytes by OCT1 (*SLC22A1*), out of hepatocytes and renal tubular epithelium by MATE1 (*SLC47A1*), and eliminated through the renal tubule cells by OCT2 (*SLC22A2*) [11–15]. All three transporters are expressed in the ovaries, hepatocytes and skeletal muscle [16] suggesting that genetic variability in metformin transport may affect metformin levels and action in these tissues of interest in PCOS.

Genetic variants in *SLC22A1* (OCT1; metformin influx transporter), *SLC22A2* (OCT2; metformin efflux transporter) and *SLC47A1* (MATE1; metformin efflux transporter) have been demonstrated to affect metformin response in patients with Type 2 diabetes [17–22]. Variants in OCT1 have been associated with reduced metformin uptake *in vitro* and *in vivo*, with elevated plasma metformin levels and decreased metformin response demonstrated in humans [18,21].

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On the contrary, *SLC47A1* (MATE1) variants result in increased response to metformin [17,18]. *SLC22A2* (OCT2) variants are associated with decreased renal metformin clearance [19,20]. In addition to metformin transport protein heterogeneity, a variant in linkage disequilibrium with *ATM* (rs11212617) has been associated with reduced HbA1C levels after metformin treatment through phosphorylation and activation of the AMP-activated protein kinase pathway [14,21]. Thus, these functional variants in metformin transporters and mediators appear to have clinical significance.

Metformin transporter pharmacogenetics has not been systematically investigated in women with PCOS. Two studies examined the role of variants in *SLC22A1* and the response to metformin in women with PCOS [22,23]. In one study, subjects received variable metformin dosing and were not extensively evaluated for important outcome parameters [5] including fasting glucose levels, glucose effectiveness, testosterone levels and weight. The second study examined one variant in *SLC22A1*, which was not associated with any outcome parameters [23]. Thus, further examination is necessary to provide insight into patients that will benefit from treatment.

We hypothesized that loss-of-function variants in *SLC22A1* would result in reduced intracellular metformin levels, and variant rs11212617 near *ATM* would result in less improvement in the critical metformin outcome parameters described previously [5]. Conversely, we hypothesized that loss-of-function variants in *SLC47A1* (MATE1) and *SLC22A2* (OCT2) would result in higher intracellular metformin levels and, subsequently, greater improvements in measured clinical outcomes. These hypotheses were tested and replicated in an independent, controlled trial of metformin administration. Results were also replicated in the metformin arm of a randomized, controlled treatment trial of metformin.

Materials & methods

Boston cohort

Subjects (n = 38) were between the ages of 18 and 40 years and diagnosed with PCOS according to the NIH criteria, which are a subset of the Rotterdam criteria: irregular menses (<9 menstrual periods/year); and clinical and/or biochemical evidence of hyperandrogenism. Clinical hyperandrogenism was defined as a Ferriman–Gallwey score greater than 9, the upper 95% confidence limit for the Boston-based control populations [24]. Biochemical hyperandrogenism was defined as an androgen level greater than the 95% confidence limits in control subjects with regular, ovulatory menstrual cycles: testosterone >63 ng/ml (2.8 mol/l), DHEAS >430 µg/dl (1.16 µmol/l) or androstenedione levels >3.8 ng/ml (0.13 nmol/l) [25]. Subjects were Caucasian (n = 21), African–American (n = 5), Asian (n = 4) and of mixed ethnicity (n = 4) and Hispanic subjects were of Caucasian (n = 1) and African–American (n = 3) ethnicity.

All subjects were otherwise healthy nonsmokers with normal thyroid and renal function, normal prolactin levels, no diabetes and a premenopausal follicular phase follicle stimulating hormone level. Nonclassic congenital adrenal hyperplasia was excluded with a follicular phase 17-OH-progesterone ≤300 µg/dl (9.1 nmol/l) [26]. Subjects were on no hormonal medication for at least 3 months and no medications that influence insulin, inflammation, or lipid levels for at least 1 month. Subjects had no plans for pregnancy during the study period.

Subjects underwent a protocol as previously described [5]. A baseline ultrasound and blood collection for estradiol and progesterone were performed at a screening visit. Subjects were observed prospectively (average: 41 days) to validate baseline menstrual cycle frequency by history. Subjects were then admitted to the Massachusetts General Hospital Human Research Center (MA, USA) at 8 AM. After a short physical exam, subjects underwent fasting blood samples and an intravenous glucose tolerance test (IVGTT), with glucose 0.3 g/kg administered at time 0 and regular human insulin 0.03 U/kg injected at 20 min [27]. At the same visit, subjects underwent a transvaginal ultrasound (Philips HD11XE, 4–8 MHz convex array transducer, Amsterdam, The Netherlands). Finally, subjects had a baseline blood sample drawn for androgens and SHBG.

After the initial visit, subjects started treatment with metformin ER 500 mg/day, with the dose increasing by 500 mg every 2 weeks to a final dose of 1500 mg/day, which was administered for a total of 12 weeks. Subjects returned every 2 weeks for anthropomorphic measurements, estradiol and progesterone levels, and a pelvic ultrasound to monitor folliculogenesis. Subjects returned for additional visits if follicle size indicated impending ovulation. Compliance was determined by questioning at the biweekly visits. After 12 weeks of metformin ER 1500 mg/day, subjects were admitted to the Massachusetts General Hospital Clinical Research Center to repeat the study as outlined above. Plasma metformin levels were measured in the fasting state at the study conclusion before the final intravenous glucose tolerance test. Clinical parameters were previously reported [5].

Virginia Commonwealth University cohort

Subjects were women with PCOS (n = 26) diagnosed by the Rotterdam criteria: irregular menstrual cycles;

biochemical hyperandrogenism with an elevated testosterone or free testosterone and/or; polycystic ovary morphology. Subjects were of Caucasian (n = 12), African–American (n = 13) and Asian (n = 1) ethnicity.

Subjects underwent an oral glucose tolerance test with glucose and insulin measured at 30-min intervals and measurement of baseline testosterone levels. Metformin 2000 mg was then administered daily for 9 months. The oral glucose tolerance test and measurement of testosterone levels were repeated on the final day of metformin treatment. During the study, subjects collected daily early morning urine for determination of pregnandedio-3-glucuronide and maintained menstrual diaries. Subjects attended study visits monthly for a compliance check and submission of urine samples. Ovulation was defined as a rise in urinary pregnandedio-3-glucuronide followed within 2 weeks or less by menstrual bleeding. Blood and/or saliva was used for DNA extraction and genotyping assays.

Reproductive medicine network cohort

DNA samples were obtained from the subgroup of women treated with metformin in a randomized trial of clomiphene citrate, metformin or the combination for the treatment of infertility [28] in the Reproductive Medicine Network (RMN) study PPCOSI (n = 131). PCOS was defined using the NIH criteria, as a subset of the Rotterdam criteria: irregular menses (<9 menstrual periods/year); and biochemical evidence of hyperandrogenism with an elevated testosterone at the enrolling site. The subjects were of Caucasian (n = 93), African–American (n = 21), Asian (n = 3) and Native American (n = 14) ethnicity, and Hispanic subjects were of Caucasian (n = 13), African–American (n = 1) and Native American (n = 13) ethnicity.

Subjects were treated with metformin ER 2000 mg for up to 30 weeks, six cycles or until they became pregnant. Weight and BMI, fasting glucose and insulin levels, testosterone levels and ovulation were recorded before treatment and at monthly visits. Progesterone levels were drawn weekly at a local laboratory to document ovulation. Results from subjects treated with metformin only were used for analysis of ovulation and testosterone levels.

Ethical approval

The study was approved by the Institutional Review Board at each enrolling center, and all subjects provided written informed consent. Race and ethnicity were self-reported at all study sites.

Assays

Serum testosterone levels in the Boston and RMN cohorts were measured using a radioimmunoassay (Coat-a-Count®, Diagnostic Products Corporation, CA, USA). Serum testosterone levels in the Virginia Commonwealth University (VCU) cohort were measured using a monoclonal ELISA assay (R&D Systems, Inc., MN, USA). SHBG was measured using a chemiluminescent enzyme immunometric assay (Immulite®, DPC, Erlangen, Germany). Insulin was measured using an immunochemiluminescent immunoassay (Immulite® 2000, DPC), with a lower LOD of 2.0 μIU/ml (14.4 pmol/l). Metformin plasma levels were measured using LC-MS/MS (NMS Labs, PA, USA) with a lower limit of detection of 0.2 μg/ml (1.55 μmol/l).

Genotyping

Genomic DNA was extracted from whole blood and amplified using PCR. Exon sequencing, including splice sites, was performed for *SLC22A1*, *SLC22A2*, *SLC47A1* and *ATM* using Sanger sequencing (Center for Computational and Integrative Biology DNA Sequencing Core, MA, USA). Sequences were examined and genotype calls were made at targeted bases for coding variants previously demonstrated to exhibit a functional effect and those previously associated with a change in metformin response or dynamics [13,14,17,18,20,29–32]. Variants in the 5′-UTR (rs2252281) and in intron 10 (rs2289669) of *SLC47A1* were sequenced based on previous data suggesting a functional effect of these variants [14,17,32,33]. Available sequences were also examined for evidence of novel polymorphisms. In addition, all DNA from the VCU cohort was analyzed using TaqMan® SNP genotyping assay reagents (Applied Biosystems®, CA, USA). A variant in *SLC47A1,* rs8065082, which was in LD (r^2 = 0.87) with rs2252281 was also analyzed.

Statistical analyses

MinMod Millenium [34] was used to analyze IVGTT data. Data were log-normalized for additional analysis. Responders and nonresponders were determined for glucose-mediated glucose disposal, baseline glucose levels, weight and testosterone levels, as previously described [5]. Comparisons of baseline characteristics between the three cohorts were made using ANOVA on ranks, where a $p < 0.05$ was considered significant. Associations between genetic variants and response to metformin were analyzed using Chi-square [35] and one-way ANOVA. To correct for multiple testing, a p-value of 0.004 was considered significant to correct for comparisons of four independent outcomes and eight evaluable variants. In addition, a separate analysis was performed for Caucasians to control for the possibility of population stratification. Other ethnicities were too few in number to perform a similar analysis. The odds ratios and p-values for the combined dis-

covery and replication groups were calculated using a Mantel–Haenszel model. Spearman rank–order correlation tests were used to examine relationships between metformin plasma levels with clinical parameters. Data are reported as mean ± standard error (SE), except where noted.

Results

The metformin dose used was higher and the duration of use was longer in the VCU and RMN cohorts. Subjects from the RMN cohort had higher glucose and insulin levels at baseline compared with the other cohorts (Table 1). The ovulation rate was slightly, but not significantly higher in the VCU cohort (75%) compared with the Boston (60%) and RMN (61%) cohorts (p = 0.8). There was no difference in ovulation, testosterone or glucose response rate in Caucasians and African–Americans within any of the cohorts (all p > 0.05).

In the Boston cohort, one subject of 38 did not carry any of the examined variants. In the RMN cohort, ten subjects did not carry any variants. In the VCU cohort all subjects carried at least one variant. No variants were found in *SLC22A2* (OCT2).

All combined results are presented in Table 2. Minor allele variants at rs683369 (L160F) were associated with less weight loss in the Boston cohort after correction for multiple testing. Heterozygote carriers of rs683369-G (L160F) gained 0.2 ± 1.4 kg compared with a weight loss of 5.3 ± 1.0 kg (p = 0.003). However, this finding was not replicated in the RMN cohort (combined p = 0.8; Table 2) and was not identified in the Caucasian subset from any group (combined p = 0.8; Supplementary Table 1). There were no additional differences in the response parameters including glucose mediated glucose disposal, fasting glucose and testosterone levels and ovulatory frequency as a function of genotype identified in any of the cohorts individually or the meta-analysis.

Metformin plasma levels

The average metformin plasma level was 0.73 µg/ml in subjects from the Boston cohort (range: 0.2–1.5 µg/ml). Subjects who ovulated had a lower mean metformin plasma level (0.6 ± 0.54 µg/ml vs 1.1 ± 0.41 µg/ml, p = 0.016). Metformin levels were not measured in the additional cohorts.

Discussion

Common genetic variants in *SLC22A1* (OCT1; metformin influx transporter), *SLC22A2* (OCT2; metformin efflux transporter), *SLC47A1* (MATE1; metformin efflux transporter) and rs11212617 in linkage disequilibrium with *ATM* are not associated with decreased glucose or testosterone levels, weight, improved glucose mediated glucose disposal or ovulation in women with PCOS. Although the data demonstrated less weight loss in carriers of rs683369-G allele (L160F) identified in the Boston cohort treated for 12 weeks with metformin, the finding was not replicated in cohorts treated for a longer duration. It is possible that weight loss is more apparent after a shorter duration of treatment, but it is more likely that the variants present in the organic cation transporters do not affect these validated clinical responses [5] in women with PCOS.

Variants in *SLC22A1, SLC22A2* and *SLC47A1* have been associated with changes in metformin pharmacokinetics and clinical response in diabetes [13,14,17,18,20,29–33]. However, the relationship between these variants and metformin has not been universal in demonstrating an HbA1C response or diabetes prevention [21,31].

Table 1. Clinical features of polycystic ovary syndrome subjects in the three cohorts.

Clinical feature	Boston cohort	RMN cohort	VCU cohort	p-value
Age (years)	29.4 ± 4.9	28.1 ± 4.2	27.9 ± 4.5	0.400
BMI (kg/m^2)	32.5 ± 1.5	34.4 ± 0.75	36.1 ± 0.9	0.176
Weight baseline (kg)	83.6 ± 5.5	92.4 ± 2.1	94.5 ± 2.2	0.144
Delta weight (kg)	-1.4 ± 0.5	-0.9 ± 0.3	-0.5 ± 1.3	0.565
Total testosterone baseline (ng/dl)	67.65 ± 8.25	58.63 ± 3.56	111.40 ± 9.49[§]	0.172
Delta testosterone (ng/dl)	-11.38 ± 4.33	-7.62 ± 3.01	6.85 ± 2.47[§]	0.603
Baseline glucose (mg/dl)	80.60 ± 1.26[‡]	92.34 ± 2.76[†,‡]	79.62 ± 1.87[†]	<0.001
Delta glucose (mg/dl)	-2.40 ± 0.90	2.75 ± 1.84	2.48 ± 0.50	0.061
Baseline insulin (uIU/ml)	9.81 ± 1.30[‡]	24.99 ± 2.72[†,‡]	9.08 ± 0.46[†]	<0.001
Delta insulin (uIU/ml)	-1.04 ± 0.96	-0.60 ± 3.61	0.24 ± 0.80	0.288

[†]Significant difference between VCU cohort and RMN cohort.
[‡]Significant difference between RMN cohort and Boston cohort.
[§]Testosterone levels were not included in the analysis because of the different assay used (see 'Materials & methods').
RMN: Reproductive Medicine Network cohort. VCU: Virginia Commonwealth University cohort.

Table 2. Frequency of functional organic ion transporter variants in women with polycystic ovary syndrome treated with metformin.

Gene	Variant	Minor allele/ ancestral allele	Protein change	Cohort	Basal glucose			Testosterone			Weight			Ovulation		
					Responder MAF	Non-responder MAF	Overall p-value	Responder MAF	Non-responder MAF	Overall p-value	Responder MAF	Non-responder MAF	Overall p-value	Responder MAF	Non-responder MAF	Overall p-value
SLC22A1	rs12208357	C/T	R61C	Boston	0.875	0.917	0.9	0.920	0.818	0.7	0.950	0.812	0.9	0.909	0.857	0.7
				RMN	0.939	0.938	–	0.938	0.950	–	0.944	0.92	–	0.940	0.947	–
	rs683369	G/C	L160F	Boston	0.800	0.885	0.8	0.903	0.667	0.8	0.905	0.735	0.9	0.804	0.867	0.4
				RMN	0.873	0.792	–	0.773	0.833	–	0.840	0.796	–	0.803	0.786	–
	rs2282143	C/T	P341L	Boston	0.977	1.0	0.5	0.979	1.0	0.5	0.972	1.0	0.3	1.0	0.958	0.2
	rs628031	A/G	M408V	Boston	0.620	0.538	0.5	0.654	0.458	0.9	0.690	0.471	0.6	0.543	0.670	0.4
				RMN	0.647	0.653	–	0.606	0.675	–	0.630	0.72	–	0.621	0.650	–
				VCU	0.600	0.714	–	0.928	0.667	–	0.615	0.615	–	0.667	0.5	–
	rs202220802	-/ATG	M420Del	Boston	0.229	0.045	0.8	0.208	0.091	0.9	0.225	0.100	0.9	0.136	0.231	0.03
				RMN	0.147	0.150	–	0.151	0.010	–	0.167	0.080	–	0.091	0.200	–
	rs34059508	G/A	G465R	Boston	0.954	1.0	0.1	0.958	1.0	0.5	0.972	0.969	0.7	0.977	0.958	0.7
				RMN	0.965	0.982	–	0.968	1.0	–	0.977	0.962	–	0.984	0.972	–
SLC47A1	rs2252281	C/T	5′-UTR	Boston	0.295	0.136	0.4	0.750	0.778	0.5	0.694	0.833	0.2	0.786	0.708	0.8
				RMN	0.380	0.405	–	0.621	0.619	–	0.605	0.611	–	0.645	0.579	–
	rs2289669	G/A	Down-stream	Boston	0.580	0.654	0.6	0.596	0.625	0.5	0.571	0.647	0.3	0.609	0.600	0.7
				RMN	0.658	0.621	–	0.690	0.667	–	0.630	0.673	–	0.719	0.619	–
				VCU	0.900	0.821	–	0.857	0.833	–	0.846	0.731	–	0.777	0.812	–
ATM	rs11212617	C/A	Intron C11orf65	Boston	0.560	0.385	0.4	0.481	0.542	0.7	0.595	0.382	0.9	0.500	0.500	0.1
				RMN	0.409	0.483	–	0.448	0.342	–	0.460	0.407	–	0.400	0.583	–

The table outlines the gene, variant, associated protein change, cohort and minor allele frequencies in Glu, T, Wt and Ov responders and nonresponders after 12 weeks of treatment with metformin (Boston cohort), up to 6 months of treatment (RMN) and 9 months of treatment (VCU). The overall p-value for the combined group was determined by a meta-analysis using a Mantel–Haenszel model.
Glu: Glucose; MAF: Minor allele frequency; Ov: Ovulatory; RMN: Reproductive Medicine Network cohort; T: Testosterone; VCU: Virginia Commonwealth University cohort; Wt: Weight.

The current study, which represents the largest population to date, and others [21,23] fail to demonstrate a relationship between organic cation transport variants and glucose, insulin and testosterone levels, a response to an oral glucose tolerance test or IVGTT, and ovulation in patients with PCOS. Thus, the data to date do not support an important effect of these variants in the response to metformin in PCOS.

Recent studies suggest that the primary glucose-lowering effect of metformin results from gut absorption [36]. Therefore, local gut and plasma metformin levels may be critical to therapeutic efficacy. Consistent with the hypothesis, plasma levels of metformin predicted ovulatory response. These findings need replication, which was not available in the current study. In addition, human studies suggesting reduced metformin action in carriers of *SLC22A1* variants hypothesize reduced transport of metformin into the intracellular space with subsequent reduction in intracellular metformin activity [13]. We and others have examined plasma metformin levels and the relationship to metformin response [37] as an inverse surrogate for intracellular metformin tissue concentrations such that lower plasma levels of metformin suggest higher intracellular metformin concentrations at target tissues of interest, particularly hepatocytes and skeletal muscle. Consistent with the absence of a relationship between *SLC22A1* variants and clinical outcomes, plasma levels of metformin were not associated with *SLC22A1* variants analyzed in the current study.

There is ethnic variability in the allele frequencies, which could have masked significant results. However, there was no difference when the Caucasian subset was analyzed independently and there was no difference in the response rate outcome parameters between ethnic groups. No variants were identified in *SLC22A2* (encodes efflux transporter OCT2), as the polymorphisms may be more prevalent in Asian populations [19,20]. There were no associations between variants in *SLC47A1* (encodes efflux transporter MATE1) or variants near *ATM* and metformin treatment response.

The importance of the replication design cannot be overemphasized. The study had 50% power to detect an effect size of 20% in a variant with at least 10% frequency and at an α level of 0.05. When a number of end points and variants are examined, the statistical chance for false positive results is also high. Therefore, increasing numbers was critical. All cohorts used for the current meta-analysis measured the same end points, using the same assays for glucose and insulin, and had careful compliance checks. The meta-analysis employed χ^2 analyses in the cohorts using responders (decrease in measured parameter) versus nonresponders (no change or increase in measured parameter). Therefore, the particular assay results from the cohorts were not directly compared, rather the change in parameters were compared, and these should have demonstrated similar trajectories between cohorts if the variants had a significant effect.

The heterogeneity between the independent cohorts, in particular the metformin treatment dosage and duration, could have masked findings. For example, the longer treatment in the VCU and RMN cohorts might mask an acute effect. The higher dose in the VCU and RMN cohorts could theoretically overcome a less effective transporter. However, the dose and duration of treatment was similar in these two cohorts and they did not replicate each other. The variants selected have demonstrated functional effects in the published literature and are more likely to yield observable outcomes. The fact that the three cohorts, or even two of the three cohorts had no replication suggests that, at least in PCOS, these variants do not robustly influence carefully chosen clinical end points.

In addition, it is presumed that mutations which determine drug response have not undergone significant evolutionary selection pressure in contrast to disease processes. It is possible that glucose parameters may be mediated by other pathways, such as those involving AMPK [38] and mitochondrial complex I [39–41], that may have redundancy. The current study may have been underpowered to detect a change in glucose parameters as others have detected changes in glycosylated hemoglobin and glucose levels [29,30,36], but no trends are demonstrated despite higher doses used in two of the three cohorts. The association between metformin transporter variants and weight has not been carefully examined in the literature. The association between variant rs683369 (L160F) variant and reduced weight loss in the Boston cohort may represent an acute effect of metformin on OCT1 transporters, as this effect was not consistent in the other cohorts treated for a longer duration. However, a trend might be expected in the other groups and was not observed.

Finally, other transporters or interactions between transporters could explain the clinical response to metformin [11,42]. It is not known whether plasma membrane monoamine transporter and OCT3 compensate for altered intracellular metformin transport by increasing metformin absorption from the GI tract [43,44] or whether genetic variation in these transporters affects metformin response. These parameters were not examined in the current study, but warrant future investigations.

Conclusion

The variants near *ATM* and nonsynonymous variants in the organic ion transporters do not consistently

explain the clinical difference in response to metformin among women with PCOS. The role of organic ion transporter protein variants and other genetic variants on metformin response in women with PCOS would benefit from large-scale, genome-wide studies.

Future perspective

The mechanism of metformin action has long been poorly understood and response to treatment variable. Continued examination of genetic variants influencing metformin transport and action and unbiased, genome-wide studies of genetic variants associated with metformin response will identify genetic markers that predict treatment response in women with PCOS. These studies will be critical for personalized treatment in women with PCOS.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/pgs-2016-0079

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Definition and significance of polycystic ovary syndrome (PCOS):
  - Polycystic ovary syndrome is the most common endocrine disorder in women of reproductive age.
  - Diabetes and impaired glucose tolerance are common sequelae of PCOS.
- Treatment of PCOS:
  - Metformin treats ovulatory dysfunction (effective in 60%), lowers testosterone levels and improves glucose-mediated glucose disposal in a subset of women with PCOS.
  - Weight loss is variable in PCOS.
- Importance of genetic variants in metformin transporters:
  - Gene variants in *SLC22A1*, *SLC22A2*, *SLC47A1*, and *ATM* rs11212617 contribute to variable metformin treatment response in diabetes.

Materials & methods

- We genotyped gene variants in *SLC22A1*, *SLC22A2* and *SLC47A1* demonstrated to affect metformin transport or clinical outcome in diabetes and a variant in *ATM* associated with metformin treatment response in diabetes.
- Genotypes were associated with carefully chosen clinical end points in women with PCOS treated with metformin.

Results

- Variant rs683369 (L160F) was associated with less weight loss in the Boston cohort ($p = 0.003$), but these results were not replicated (combined $p = 0.8$).
- There were no additional differences in glucose parameters, testosterone levels, or ovulatory frequency as a function of genotype in the cohorts individually or in the meta-analysis.

Conclusion

- The current study represents the largest PCOS population examined to date.
- The variants in metformin transporters and *ATM* are not associated with treatment outcome in PCOS.
- The tested variants in metformin transporters have no robust, consistent effect on metformin response in PCOS.

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• of interest; •• of considerable interest

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Case Report

Raspberry Leaf and Hypoglycemia in Gestational Diabetes Mellitus

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BACKGROUND: Raspberry leaf is commonly consumed by pregnant women. Hypoglycemic effects have been documented with other species within the plant family. Whether raspberry leaf affects glycemic control in gestational diabetes mellitus (GDM) is unknown.

CASE: A 38-year-old nulliparous woman with GDM developed hypoglycemia requiring lowered insulin dose after consuming raspberry leaf tea at 32 weeks of gestation. The temporal relationship was confirmed by the patient's self-withdrawal and reintroduction of the herb. Fetal surveillance and growth were reassuring. A cesarean delivery was performed at 39 weeks of gestation. The neonate did not experience hypoglycemia or other complications. Placental biopsy revealed normal findings.

CONCLUSION: Consumption of raspberry leaf may lead to reduced insulin requirements in GDM. Women with GDM should be cautioned about its use and their glucose levels more closely monitored.

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Teaching Points

1. Clinicians should interview pregnant patients about use of alternative medicine.
2. Use of raspberry leaf may lead to hypoglycemia and lowering of insulin requirements in gestational diabetes mellitus treated with insulin.
3. Women with gestational diabetes mellitus should be cautioned about use of raspberry leaf, and their glucose levels should be more closely monitored.

The use of alternative medicine in pregnancy has gained interest from both patients and health care providers. Among U.S. pregnant women, 29% reported herbal medicine use,[1] and 93.9% of midwives reported recommending alternative medicine to their pregnant patients.[2] Raspberry leaf (*Rubus idaeus,* or *Rubus strigosus,* plant family *Rosaceae*) is a commonly used herbal product during pregnancy[3] on the belief that it could shorten labor.[4] Based on our MEDLINE search (1964–2016) with terms "glucose" (MeSH term), "diabetes, gestational" (MeSH term), and raspberry (all fields), "rubus" (MeSH term), "rubus idaeus" (all fields), "rubus strigosus" (all fields), the effect of raspberry leaf on glycemic control in women with gestational diabetes (GDM) has never been documented. We present a case of hypoglycemia and reduced insulin requirements in a woman with GDM using raspberry leaf tea in the third trimester.

CASE

A 38-year-old Asian woman, gravida 2 para 0010, with GDM presented at her routine prenatal visit at 32 weeks of gestation and reported hypoglycemic episodes. Gestational diabetes mellitus was suspected at 10 weeks of gestation when her glucose was positive with urine dipstick. Her 50-g glucose challenge yielded 199 mg/dL (1 hour). Hemoglobin A1c drawn on the same day was 5.4%, suggesting she did not have pre-existing diabetes. Her 3-hour glucose challenge results were 84 mg/dL (fasting), 184 mg/dL (1 hour), 156 mg/dL (2 hours), and 94 mg/dL (3 hours). Her glucose had been under excellent control with medical nutrition therapy, neutral protamine Hagedorn insulin (NPH), and preprandial insulin lispro (dosed by carbohydrate counting). Self-monitoring of glucose was performed at fasting, before and after meals, and at bedtime. Her insulin requirements had been increasing throughout the pregnancy. At 13 weeks of gestation, her lispro requirement was 1 unit/15 g of carbohydrate without any NPH. Her last dose increase was at 30 weeks of gestation (Table 1), 2 weeks before her report of hypoglycemic episodes (to NPH 22 units twice daily and lispro 1 unit/3 g of carbohydrates). She reported no change in dietary or physical activity habits other than the addition of two servings of raspberry leaf tea (dried red raspberry leaf as the only ingredient) 3 days prior (at 32 2/7 weeks of gestation). She initiated the raspberry leaf tea in hopes that it might shorten her labor. Examination of her glucose logs revealed good

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Table 1. Temporal Relationship Between Raspberry Leaf Consumption and Glycemic Control (From the Patient's Glucose and Food Log)

Gestational Week	Insulin Dose*	Raspberry Leaf Tea Consumption†	Glucose Reading‡ (mg/dL)
30 0/7 to 32 1/7	NPH 22 units; lispro 1 unit/3 g carb	No	At goal
32 2/7		Yes	At goal
32 3/7		Yes	2 h PP **52–66;** bedtime **48**
32 4/7		Yes	2 h PP **54–63;** bedtime **55**
32 5/7§	Dose change: NPH 22 units; lispro 1 unit/4 g carb	Yes	At goal
32 6/7 to 33 2/7§		Yes	At goal
33 3/7		No	1 h PP 118–145
33 4/7		No	2 h PP **155–163**
33 5/7		Yes	2 h PP 114–144
33 6/7		Yes	At goal
34		No	1 h PP 128–147
34 1/7		No	2 h PP **157–176**
34 2/7	Dose change: NPH 22 units; lispro 1 unit/3 g carb	No	At goal
34 4/7§ to 35 3/7		No	At goal
35 4/7	Dose change: NPH 22 units; lispro 1 unit/4 g carb	Yes	At goal
Remainder of pregnancy	Dose gradually changed to NPH 18 units; lispro 1 unit/5 g carb for morning meals, 1 unit/4 g carb for afternoon and evening	Yes	At goal

NPH, neutral protamine Hagedorn; carb, carbohydrate; PP, postprandial, 1- or 2-hour postprandial as indicated (patient alternated between checking 1-hour and 2-hour postmeal values).
Bold indicates hypoglycemia or hyperclycemia.
* NPH was administered twice daily at the listed dose.
† Raspberry leaf consumed was two servings per day.
‡ Glycemic goal was less than 90 mg/dL (premeal), less than 130 mg/dL (1 hour), less than 120 mg (2 hours).
§ Day of prenatal visit or fetal surveillance.

glycemic control since the last insulin adjustment 2 weeks prior, and hypoglycemia was experienced only since the addition of raspberry leaf tea with 2-hour postprandial glucose of 52–66 mg/dL and bedtime glucose values as low as 48 mg/dL. She reported tachycardia and mild diaphoresis with her hypoglycemia, which were self-treated with orange juice.

Fetal surveillance was reassuring with moderate variability, present accelerations, and absent decelerations. There was no evidence of uteroplacental insufficiency; however, weekly fetal surveillance was recommended as a result of concern about the hypoglycemic episodes. Prandial insulin was decreased to lispro 1 unit/4 g of carbohydrates.

At 33 2/7 weeks of gestation, a biophysical profile was performed without a nonstress test, again with reassuring findings. The biophysical profile score was 8 of 8. The ultrasonogram revealed a viable, singleton intrauterine fetal gestation in cephalic position with normal amniotic fluid, four quadrant amniotic fluid index of 144 mm, placental grade I, and appropriate fetal growth (66th percentile). The patient reported that the hypoglycemic episodes had resolved.

On the next prenatal visit at 34 4/7 weeks of gestation, the patient reported she had discontinued the raspberry leaf tea twice (Table 1), because she suspected that its use may have led to the hypoglycemia and reduced insulin needs. Each time on the day after abstaining from raspberry leaf tea, she experienced postprandial hyperglycemia (Table 1) using the same dose of lispro. She self-adjusted her prandial insulin coverage to lispro 1 unit/3 g of carbohydrate to prevent further hyperglycemia.

Over the remaining course of her pregnancy, the patient resumed drinking two servings of raspberry leaf tea per day. Her lispro was adjusted to an insulin-to-carbohydrate ratio of one to five in the morning and one to four in the afternoon with NPH 18 units twice daily. Repeat ultrasonography at 37 and 39 weeks of gestation revealed normal findings. One week before delivery, her NPH was decreased to 18 units at bedtime only. Her glucose was well controlled throughout the remainder of the pregnancy. Her weekly fetal surveillance tests remained reassuring.

At 39 weeks of gestation, the patient underwent labor augmentation with oxytocin as a result of prolonged rupture of membranes and meconium-stained amniotic fluid. A cesarean delivery was performed because of active-phase arrest in the setting of suspected chorioamnionitis, which was treated with intrapartum antibiotics. There was no intrapartum hyper- or hypoglycemia in the

mother. The patient delivered a live female neonate weighing 3,490 g (75th percentile for gestational age)[5] with Apgar scores of 9 and 9 at 1 and 5 minutes, respectively. The neonate did not experience hypoglycemia or other complications but did receive prophylactic antibiotics. Biopsy of the placenta revealed a term placenta and fetal membranes with meconium-laden macrophages but no additional abnormalities. Both the mother and neonate were discharged home after routine postpartum care. The mother's 2-hour glucose challenge at her 8-week postpartum visit yielded glucose of 79 mg/dL (fasting), 192 mg/dL (1 hour), and 148 mg/dL (2 hours).

DISCUSSION

During pregnancy, placental production of various anti-insulin hormones, cytokines, and growth factors[6] exacerbate insulin resistance.[7] Women with GDM using insulin typically have progressive increments in insulin need as pregnancy advances.[8] Falling insulin requirements have been associated with placental dysfunction, preeclampsia, small-for-gestational-age neonates, and a higher incidence of neonatal intensive care admissions.[9]

Our case describes the occurrence of reduced insulin requirements as gestation advances not resulting from placental insufficiency. We initially did not suspect the patient's self-medication with raspberry leaf could have led to hypoglycemia because such effects have not been documented in the literature. However, using the Naranjo algorithm[10] to assess causality, use of raspberry leaf tea probably had led to the hypoglycemic episodes in this case. The hypoglycemic effects followed a reasonable temporal sequence after its use and were confirmed by the patient's self-withdrawal and reintroduction of the herb. In addition, alternative causes of hypoglycemia have been ruled out. To wit, the patient reported no changes in physical activity or dietary habits. Her insulin dose had been stable for 2 weeks before the hypoglycemia. The placenta was grossly normal on inspection as well as microscopically.

There is a growing use of complementary and alternative medicine during pregnancy. In the United States, 29% of pregnant women reported self-medication with herbal medicine.[1] Most patients assume that herbal therapy has the advantage of being "natural." However, there are often insufficient efficacy and safety data for their use.[13]

Raspberry leaf (*R ideus*) has been used during pregnancy primarily on the belief that it can shorten labor,[4] but available studies have shown no difference in the need for labor augmentation, instrument-assisted births, or duration of labor.[4,12]

The risks associated with raspberry leaf have not been well studied. Blackberry leaves (*Rubus fructicosis*) and raspberry leaves *(R ideus)* belong to the same plant family, Rosaceae, and may have similar chemical properties.[13] Hypoglycemic effects of blackberry leaves have been documented in animal models and may be relevant to raspberry leaves. In diabetic rats, *R fructicosis* extract significantly reduced glucose 2 hours after a single oral dose ($P<.001$) and persisted up to 6 hours postdose.[14] On continuous administration for 9 days, *R fructicosis* reduced blood glucose to normal levels in diabetic rats ($P<.001$). In another study, supplementation of rubus extract in rats on a standard diet increased adipose tissue lipolytic activity and decreased plasma glucose.[15] Decreased insulin concentrations were also seen in female rats, suggesting increased insulin sensitivity.

The extent to which hypoglycemic effects of blackberry leaves can be extrapolated to raspberry leaf tea in the amount typically consumed by pregnant women is unknown. We did not initially suspect the patient's hypoglycemia was the result of use of raspberry leaf tea because literature on its glycemic effect is nonexistent. Additional fetal surveillance was performed as a result of the unexpected reduction in insulin requirements. We suggest that in women with GDM using insulin, use of raspberry leaf may require closer glucose monitoring to prevent inadvertent hypoglycemic episodes. It will be prudent to interview patients about use of alternative medicine when hypoglycemic episodes occur. Given the paucity of data supporting the use of raspberry leaf in shortening labor, women with GDM should be cautioned about its use. For patients who prefer nondrug options and whose GDM remain uncontrolled with medical nutrition therapy, prospective clinical trials may be considered to evaluate the role of raspberry leaf in GDM.

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Divergent effects of a combined hormonal oral contraceptive on insulin sensitivity in lean versus obese women

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Objective: To evaluate the effects of a commonly used combined hormonal oral contraceptive (OC) on carbohydrate metabolism in obese as compared with lean women.
Design: 6-month prospective study.
Setting: Clinical research center at an academic medical center.
Patient(s): Premenopausal nondiabetic women with body mass index <25 kg/m^2 (n = 15) or >30 kg/m^2 (n = 14).
Intervention(s): Ethinyl estradiol (35 μg) and norgestimate (0.18/0.215/0.25 mg) for 6 cycles.
Main Outcome Measure(s): Insulin sensitivity by frequent sampling intravenous glucose tolerance test; other indices of insulin sensitivity (homeostatic model assessment of insulin sensitivity index [ISI HOMA], the Matsuda index); fasting lipid panel.
Result(s): Insulin sensitivity changed from 6.62 ± 3.69 min^{-1}/mIU/L (baseline) to 8.23 ± 3.30 min^{-1}/mIU/L (6 months) in lean women, and from 4.36 ± 2.32 to 3.82 ± 2.32 min^{-1}/mIU/L in obese women. Divergent effects on insulin sensitivity were also observed with ISI HOMA and the Matsuda index. Low-density lipoprotein increased by approximately 20 mg/dL in both the lean and obese groups.
Conclusion(s): Lean and obese women exhibit differential changes in insulin sensitivity when given 6 months of a commonly used oral contraceptive. The mechanisms of these differences and whether these divergent effects persist in the long term require further investigation. (Fertil Steril® 2011;96:353–9. ©2011 by American Society for Reproductive Medicine.)

Key Words: Carbohydrate metabolism, cholesterol, insulin resistance, obesity, oral contraceptives

The combined hormonal oral contraceptive (OC) pill is the most commonly used contraceptive method due to its effectiveness and reversibility (1, 2). Oral contraceptives, especially those with high estrogen doses (≥50 μg ethinyl estradiol), may be associated with alterations in carbohydrate metabolism (3, 4). With ethinyl estradiol at lower doses, OCs probably have limited effects on carbohydrate metabolism in normal weight women (5). However, no information is available regarding the effects among obese women (5).

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The prevalence of obesity and overweight is increasing worldwide. Obesity is associated with insulin resistance, impaired glucose tolerance, and increased risk of diabetes (6). Hence, even a small degree of worsening insulin sensitivity with OC use may be of clinical relevance for obese women.

We compared the effects of a commonly used combined hormonal OC containing 35 μg of ethinyl estradiol and 0.18/0.215/0.25 mg of norgestimate on carbohydrate metabolism in obese and lean women. We studied this particular OC because it is one of the most commonly used (2) and because the safety profile of the progestin, norgestimate, has been established, with previous reports suggesting no significant worsening of fasting insulin, glucose, or glycosylated hemoglobin in nonobese women (7). We hypothesized that combined hormonal OC affects insulin sensitivity differently in lean versus obese women. In addition, we evaluated the effects of this OC on blood pressure and lipid profile in obese versus lean women.

MATERIALS AND METHODS

Patients

Premenopausal women aged 18 to 40 years were enrolled in the study. The obese women had a body mass index (BMI) >30 kg/m^2, and the lean women had a BMI <25 kg/m^2. Women with the following characteristics were

TABLE 1

Baseline demographic and clinical characteristics of the study patients.

Parameter	Lean women (n = 15)	Obese women (n = 14)	*P* value
Age (y)	21.4 ± 2.3	22.6 ± 5.4	.4357
BMI (kg/m^2)	21.3 ± 1.8	37.1 ± 6.7	<.0001
No. of metabolic syndrome risk factors[a]			.0017
0	11	2	
1	4	5	
2	0	4	
3+	0	3	
Race			
Caucasian	10 (66.7)	7 (50)	.1956
Non-Caucasian	5 (33.3)	7 (50)	

Note: Values were mean ± standard deviation for continuous variables, or number (%) for categorical variables. BMI = body mass index.
[a] *P* value was from chi-square test comparing the number of participants with and without at least two metabolic syndrome risk factors present at baseline.

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excluded: [1] diabetes determined by fasting glucose or a 2-hour glucose tolerance test (OGTT); [2] contraindications to OC use (e.g., history of thromboembolism, coronary/ cerebrovascular events, prolonged immobilization, blood pressure ≥160/100 mm Hg, age ≥35 years and smoker of ≥20 cigarettes/day, migraines, malignancies, or hepatic diseases); [3] use of systemic hormonal contraceptives, insulin sensitizers, antihyperlipidemic drugs, antihypertensives, or glucocorticoids within 3 months; [4] pregnancy or lactation; or [5] actively attempting weight loss (>2 kg of weight loss in the previous month). All of the women were cycling normally.

Before the study procedures, all participants provided signed, informed consent. The study was approved by the Virginia Commonwealth University institutional review board, and was registered at clinicaltrials.gov (NCT00205504). None of the authors had any conflict of interest.

Study Procedures

All evaluations were performed in the follicular phase, confirmed by a serum progesterone concentration <2 ng/mL. The participants were admitted to the general clinical research center after a 12-hour fast. On day 1, their blood pressure, anthropometric measurements, comprehensive metabolic panel, fasting lipid profile, and serum fasting insulin and glucose concentrations were obtained. Absence of pregnancy was confirmed by a urine pregnancy test. The participants underwent a 2-hour OGTT with 75 g glucose, and blood samples were collected every 15 minutes for the determination of serum glucose and insulin concentrations.

On day 2, after a 12-hour fast, the participants underwent the modified frequent sampling intravenous glucose tolerance test (FSIVGTT) (8–10). At time 0, 300 mg/kg dextrose was administered intravenously over 1 minute, and insulin at 0.03 IU/kg was administered similarly 20 minutes later. The serum glucose and insulin concentrations were obtained at 29 time points over 3 hours (10). The women received supplies of 35 μg of ethinyl estradiol and 0.18/0.215/0.25 mg of norgestimate (Ortho Tri-Cyclen; Ortho-McNeil Pharmaceuticals) and were instructed to start administering the OC that day. Proper use of the OC was explained. The women were instructed not to modify their dietary and physical activity habits from baseline during the study period.

During week 12 (third cycle of OC), the women returned for testing between day 5 and 7 of the hormone-free week, to minimize the effect of progestins on insulin sensitivity. The assessments for this visit were the same as day 1. Medication compliance was verified by interview and pill counts. During the last week of cycle 6 (6 months), the women returned between day 5 and 7 of the hormone-free week for repeat assessments, as performed in days 1 and 2.

Laboratory Assays

See the laboratory assays in the Supplemental Materials and Methods, available online.

Metabolic Syndrome Risk Factors

The number of metabolic syndrome risk factors at baseline in both lean and obese women were evaluated to serve as a general indicator of metabolic risk. The National Cholesterol Education Program definition of the metabolic syndrome was used (11).

Insulin Sensitivity and Indices of Insulin-Glucose Dynamics

Glucose-insulin dynamics during FSIVGTT were analyzed using the Minimal Model Identification software (MINIMOD, version 6.02) (12). The FSIVGTT yields quantitative determinations of [1] tissue insulin sensitivity (Si); [2] acute insulin response to glucose (AIRg), which addresses the adequacy of insulin secretion; [3] disposition index (DI), or AIRg * Si, which is a composite measure of insulin secretion and action; and [4] glucose effectiveness (Sg), the capacity of glucose to mediate its own disposal independent of insulin (12).

We analyzed the insulin and glucose incremental areas under the curve (AUC) upon OGTT by the trapezoidal rule after subtracting baseline values. We assessed incremental AUCs because fasting baseline values of insulin and glucose were already separately presented, and incremental AUCs reflect changes in response to glycemic loads (13). The glucose and insulin values during OGTT were used to calculate the Matsuda insulin sensitivity index (14). The homeostatic model assessment insulin sensitivity index (ISI HOMA) (15) was calculated from fasting glucose and insulin concentrations.

Statistical Analysis

The primary outcome was the mean change in insulin sensitivity (Si from FSIVGTT) from baseline to 6 months among obese versus lean women. Secondary outcomes were incremental $AUC_{glucose}$, incremental $AUC_{insulin}$, fasting glucose and insulin concentrations, insulin sensitivity as measured by ISI HOMA and Matsuda indices, systolic and diastolic blood pressure, and lipid and anthropometric parameters. Normal distributions were determined by normal probability plots. Continuous variables were presented as mean values ± standard deviation. Results not normally distributed were log-transformed for statistical analyses and, after back-transformation, were reported in their original units as geometric means with 95% confidence intervals.

Baseline comparisons between obese and lean women were performed via Student's *t*-test, and Welch analysis of variance (ANOVA) tests if unequal variances were observed. Proportions were compared by Pearson chi-square tests. We evaluated the mean change with OC administration in each parameter within all of the women, and within the obese and lean groups separately, using a paired *t*-test.

For the primary outcome of interest, the change in Si after 6 months of OC was compared between the obese and lean women using a repeated measures analysis, testing for interaction between the Si time trends and the baseline

TABLE 2

Effects of the combined oral contraceptive pill on glucose metabolism, blood pressure, and lipid parameters in lean and obese women.

Parameter	Lean women (n = 15)		Obese women (n = 14)		*P* value (baseline comparisons between groups)[a]	*P* value (comparisons of OC effects between groups)[b]
	Baseline	6 mo	Baseline	6 mo		
Glucose metabolism						
Fasting glucose (mg/dL)	81 ± 4.0	82 ± 5.2	85 ± 4.8	86 ± 5.1	.0249	.9837
Fasting insulin (μIU/mL)	3.6 ± 1.25	3.7 ± 3.40	7.3 ± 5.99	8.7 ± 3.28	.0369[c]	.4627
Incremental AUC insulin$_{0-120}$ (μIU/mL • min)[d]	3,762 ± 2,453.1	3,439 ± 2,501.5	4,274 ± 2,370.0	4,301 ± 2,416.7	.4889	.5102
Incremental AUC glucose$_{0-120}$ (mg/dL • min)[d]	2,917 ± 2,274.3	4,208 ± 2,325.0	2,686 ± 2,197.1	2,676 ± 2,246.2	.8090	.2337
Fructosamine (μmol/L)	319 ± 79.7	302 ± 75.8	270 ± 94.0	320 ± 65.7[e]	.1393	.1284
Si (min^{-1}/mIU/L)[f]	6.62 ± 3.69	8.23 ± 3.30	4.36 ± 2.32	3.82 ± 3.12	.0607	.0494
AIRg (mIU • L^{-1} • min)[f]	341.6 ± 209.85	313.5 ± 381.34	803.7 ± 411.82	801.4 ± 346.65	.0013[c]	.6878
Sg (1,000 • min^{-1})ψ[f]	29.2 (22.6–37.8)	26.4 (18.8–36.9)	25.8 (19.6–33.9)	18.1 (13.1–25.1)	.4638	.3502
Disposition index (AIRg • S_i)[f]	2,062 ± 1,336.1	2,080 ± 1,657.1	3,077 ± 1,905.9	2,591 ± 1,558.9	.1067	.4288
ISI HOMA[g]	1.56 ± 0.501	2.05 ± 0.705	1.01 ± 0.574	0.63 ± 0.659[h]	.0088	.0128
Matsuda index[d]	11.8 ± 5.02	13.2 ± 4.79	7.7 ± 3.80	6.0 ± 4.54	.0188	.0227
Cardiovascular and anthropometric risk factors						
Systolic blood pressure (mm Hg)	105.7 ± 8.6	106.5 ± 12.2	122.1 ± 3.2	123.5 ± 11.7	.0010	.4826
Diastolic blood pressure (mm Hg)	68.0 ± 3.7	66.5 ± 6.8	74.4 ± 1.8	73.4 ± 6.6	.0173	.4069
BMI (kg/m^2)	21.3 ± 1.8	21.4 ± 4.9	37.1 ± 6.7	37.6 ± 4.7	< .0001	.1932
Waist circumference (cm)	70.2 ± 4.1	70.9 ± 10.7	100.0 ± 14.4	101.0 ± 10.5	< .0001	.5643
Waist-to-hip ratio	0.73 ± 0.027	0.73 ± 0.057	0.79 ± 0.059	0.81 ± 0.057	.0016	.8864
Total cholesterol (mg/dL)	155 ± 30.2	181 ± 29.1[i]	164 ± 23.3	194 ± 28.1[i]	.3613	.3857
LDL (mg/dL)	84 ± 24.3	104 ± 26.1[h]	95 ± 18.4	113 ± 25.2[j]	.1607	.2121
Triglycerides (mg/dL)	72 ± 52.0	106 ± 52.9[j]	72 ± 50.3	83 ± 51.1	.9711	.1179
HDL (mg/dL)	53 ± 10.0	61 ± 12.8[e]	51 ± 10.7	58 ± 12.4[i]	.5306	.9563
Total/HDL cholesterol ratio	3.0 ± 0.57	3.1 ± 0.78	3.3 ± 0.71	3.5 ± 0.75	.1281	.1699

Note: Values are mean ± standard deviation, or geometric mean (95% confidence interval) when indicated by the symbol ψ. AIRg = acute insulin response to glucose; AUC = area under the curve; BMI = body mass index; HDL = high-density lipoprotein; ISI HOMA = homeostatic model assessment of insulin sensitivity index; LDL = low-density lipoprotein; OC = oral contraceptive; Si = insulin sensitivity; Sg = glucose effectiveness.

[a] Baseline comparisons between groups performed by independent Student's *t*-test (unless otherwise indicated for unequal variances).

[b] Comparisons of oral contraceptive effects between lean and obese groups, performed by repeated measures analysis of variance (ANOVA): Time × Obesity status.

[c] Baseline comparisons between groups performed by Welch analysis of variance (ANOVA) test, as appropriate for unequal variances.

[d] Area under the curve (AUC) insulin and glucose, and Matsuda index of insulin sensitivity were obtained via oral glucose tolerance test.

[e] $P < .05$ for comparison between baseline and 6 months within group, performed with paired *t* tests.

[f] Estimates of insulin sensitivity (Si), acute insulin response to glucose (AIRg), glucose effectiveness (Sg), and disposition index (product of AIRg and Si) were derived from frequently sampled intravenous glucose tolerance test.

[g] Insulin sensitivity assessed by the homeostatic model assessment (ISI HOMA) were derived from fasting glucose and insulin values.

[h] $P < .005$ for comparison between baseline and 6 months within group, performed with paired *t* tests.

[i] $P \leq .001$ for comparison between baseline and 6 months within group, performed with paired *t* tests.

[j] $P < .01$ for comparison between baseline and 6 months within group, performed with paired *t* tests.

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obesity status. Changes in the secondary outcomes of interest between the two groups were analyzed similarly.

Based on a previous cross-sectional study on $AUC_{glucose}$ among nonobese and obese women taking oral contraceptives (16), 14 women per group were needed to achieve a power of 80%. Statistical analysis was performed using JMP 8.0 (SAS Institute Inc.). $P<.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

A total of 48 lean and obese women provided informed consent for the study. Of these, four women did not meet inclusion criteria, nine did not attend their first study appointment, one participant was lost to follow up after day 1, and four women withdrew from the protocol shortly after the initial baseline studies (two for personal reasons, one for fear of venipuncture, and one for dizziness after one dose of OC). One obese participant was withdrawn by the investigator due to protocol violation (the participant began a weight loss program during the study). In all, 29 women (15 lean and 14 obese women) completed the study.

At baseline, the lean and obese women were demographically similar (Table 1), but blood pressure, BMI, waist-to-hip ratio, and number of metabolic syndrome risk factors were all significantly higher in the obese group (Tables 1 and 2). As expected, the obese group was more insulin resistant at baseline (as assessed by Si, ISI HOMA, and the Matsuda index) (see Table 2). Obese women also exhibited significantly increased AIRg, possibly a compensation to reduced insulin sensitivity.

Effects of OC in All Participants

When all of the women were analyzed together, OC use for 6 months did not affect any insulin sensitivity parameters (Si, ISI HOMA, and Matsuda index), nor it have any significant effects on insulin or glucose homeostasis (incremental $AUC_{glucose}$ and $AUC_{insulin}$, AIRg, DI, and Sg) (Table 3). Systolic and diastolic blood pressure, BMI, waist circumference, and waist-to-hip ratio after 6 months of the OC also were not statistically significantly different from baseline (see Table 3). However, OC use statistically significantly increased the levels of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides (see Table 3). In particular, LDL increased by 20% (from 90 to 108 mg/dL, $P<.001$) over the 6 months of OC administration.

Effect of Baseline Obesity Status on Change in Insulin Sensitivity during OC Use

Use of the OC for 6 months resulted in divergent effects on Si as measured by FSIVGTT in lean versus obese women ($P=.0494$ for interaction between obesity status and Si time trend; Fig. 1 and Table 2). Other measures of insulin sensitivity, the Matsuda index ($P=.0227$) and ISI HOMA ($P=.0128$), all showed the same

TABLE 3

Effects of the combined oral contraceptive pill on glucose metabolism and cardiovascular parameters in all of the women.

Parameter	Baseline	24 wk	*P* value
Glucose metabolism			
Fasting insulin (μIU/mL)	5.5 ± 4.7	6.3 ± 3.8	.0833
Fasting glucose (mg/dL)	83 ± 4.8	83.7 ± 5.4	.5335
Incremental AUC $insulin_{0–120}$ (μIU/mL • min)[a]	4,070 ± 2,428	3,980 ± 1,934	.9912
Incremental AUC $glucose_{0–120}$ (mg/dL • min)[a]	2,828 ± 2,005	3,506 ± 2,344	.1178
Fructosamine (μmol/L)	295 ± 88.9	312 ± 70.2	.4693
Si (min^{-1}/mIU/L)[b]	5.36 ± 3.18	5.98 ± 4.01	.4198
AIRg (mIU • L^{-1} • min)[b]	579 ± 394.5	572 ± 451.1	.5278
Sg (1,000 • min^{-1})[b]	32.0 ± 27.3	24.7 ± 10.1	.2151
Disposition index (AIRg • S_i)	2,582 ± 1,710	2,401 ± 1,518	.2891
ISI HOMA[c]	1.25 ± 0.58	1.28 ±1.18	.3142
Matsuda index[c]	9.6 ± 4.83	9.0 ± 6.38	.3719
Cardiovascular and anthropometric risk factors			
Systolic blood pressure (mm Hg)	113 ± 13.9	116 ± 14.1	.2552
Diastolic blood pressure (mm Hg)	71 ± 7.2	70 ± 7.0	.4490
BMI (kg/m^2)	28.8 ± 9.3	29.5 ± 9.5	.1234
Waist circumference (cm)	83 ± 19.4	86 ± 18.4	.2148
Waist-to-hip ratio	0.75 ± 0.08	0.77 ± 0.06	.2270
Total cholesterol (mg/dL)	160 ± 27.4	188 ± 31.0	<.0001
LDL (mg/dL)	90 ± 22.3	108 ± 27.6	<.0001
Triglycerides (mg/dL)	73 ± 39.9	95 ± 47.6	.0049
HDL (mg/dL)	52 ± 10.4	60 ± 15.0	<.0001
Total/HDL cholesterol ratio	3.1 ± 0.67	3.3 ± 0.87	.0604

Note: Values were mean ± standard deviation. AIRg = acute insulin response to glucose; AUC = area under the curve; BMI = body mass index; HDL = high-density lipoprotein; ISI HOMA = homeostatic model assessment of insulin sensitivity index; LDL = low-density lipoprotein; Si = insulin sensitivity; Sg = glucose effectiveness.

[a] Area under the curve (AUC) insulin and glucose, and Matsuda index of insulin sensitivity were obtained via oral glucose tolerance test.

[b] Estimates of insulin sensitivity (Si), acute insulin response to glucose (AIRg), glucose effectiveness (Sg), and disposition index (product of AIRg and Si) were derived from frequently sampled intravenous glucose tolerance test.

[c] Insulin sensitivity assessed by the homeostatic model assessment (ISI HOMA) was derived from fasting glucose and insulin values.

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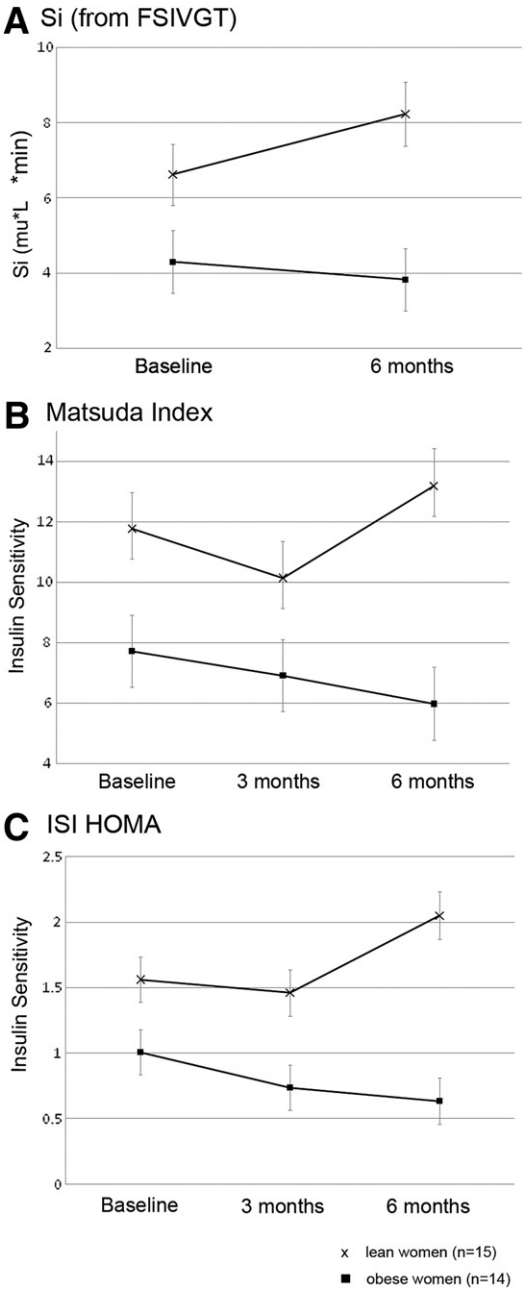
FIGURE 1

Insulin sensitivity assessments during 6 months of oral contraceptive administration in lean and obese groups (mean ± standard error of the mean). (**A**) Insulin sensitivity (Si) obtained from frequently sampled intravenous glucose tolerance test (FSIVGTT) at baseline and 6 months. (**B**) Matsuda index of insulin sensitivity obtained from oral glucose tolerance test at baseline, 3 and 6 months. (**C**) Insulin sensitivity index by homeostatic model assessment (ISI HOMA), using fasting glucose and insulin concentrations at baseline, 3 and 6 months. Lean and obese women exhibited statistically significantly different time trends in Si ($P=.0494$), Matsuda index ($P=.0227$), and ISI HOMA ($P=.0128$), during the 6 months of oral contraceptive use.



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divergent effects, suggesting that these results were internally consistent. There were no statistically significant differences between the lean and obese women in changes in AIRg, Sg, or DI during the 6 months of OC use (see Table 2). Fasting glucose, fasting insulin, incremental $AUC_{insulin}$, and incremental $AUC_{glucose}$ also did not change differentially between obese and lean women during the period of OC administration (see Table 2). Although the effect of OC on fructosamine was not statistically significantly different between the lean and obese groups, when the 6-month values were compared to baseline, fructosamine showed a statistically significant increase only in obese women (from 270 to 320 μmol/L, $P=.0449$), which suggests that the average glucose levels in obese women increased during OC administration.

Effects of OC on Cardiovascular Risk Factors in Lean and Obese Groups

Use of the OC did not change the systolic and diastolic blood pressure, BMI, waist circumference, or waist-to-hip ratio within either group (see Table 2). However, OC use statistically significantly increased the total cholesterol level in both groups. The level of LDL cholesterol increased in both the lean (+19.5 ± 14.5 mg/dL, $P=.0002$) and obese (+17.3 ± 19.9 mg/dL, $P=.0064$) women. As expected, the level of HDL cholesterol also increased in both groups (+7.5 ± 11.0 mg/dL [$P=.024$] in lean women and +7.6 ± 4.8 mg/dL [$P<.0001$] in obese women). Triglyceride levels showed a statistically significant increase only in lean women (+34.2 ± 44.5 mg/dL, $P=.0129$). There were no differential changes in any of these parameters between lean and obese women.

Discussion

We examined the effects of a commonly used combined hormonal OC on carbohydrate metabolism in obese versus lean women. Use of an OC for 6 months appeared to have divergent effects on insulin sensitivity in lean women as compared with obese women. This divergence was seen with all measures of insulin sensitivity assessed (Si, Matsuda index, and ISI HOMA).

There have been decades of research on the effects of combined OCs on carbohydrate metabolism (5, 17). The earliest studies evaluated combined OCs that had a relatively high estrogen dose (3, 4) or had progestins with higher androgenicity than those commonly used today (18). Studies performed with lower-dose estrogen and less androgenic progestins have suggested that these OCs have little to no effect on carbohydrate metabolism (7). Using the same OC agent used in our study (35 μg of ethinyl estradiol and 0.18/0.215/0.25 mg of norgestimate: Ortho Tri-Cyclen), Burkman et al. (7) reported no statistically significant changes in fasting glucose or insulin levels, or glycosylated hemoglobin in a 2-year study conducted in 1,783 healthy women. Similarly, a study of the monophasic formulation of this combined OC (35 μg of ethinyl estradiol/0.25 mg of norgestimate) in 42,022 women (342,348 menstrual cycles) suggested little effect on lipid metabolism or fasting glucose (19). However, neither of these studies directly evaluated insulin sensitivity. Moreover, studies in obese or overweight women, who are at higher risk of metabolic derangements, have been lacking, as reported in the recent Cochrane analysis (5).

Our study observed a differential effect on insulin sensitivity between obese and lean women when given a commonly used combined OC. Our findings are supported by previous evidence suggesting that baseline insulin sensitivity may affect changes in insulin sensitivity during OC administration. In women with and without prior gestational diabetes who were using OCs containing 30 μg

of ethinyl estradiol and triphasic levonorgestrel, insulin sensitivity worsened more in women with a history of gestational diabetes (20). One cross-sectional study reported that obese women taking OCs had higher $AUC_{glucose}$ during OGTT as compared with lean women (16). Finally, an ethinyl estradiol–cyproterone acetate OC worsened $AUC_{insulin}$ in obese (21), but not lean (22) women with polycystic ovary syndrome. To our knowledge, ours is the first prospective study to describe differential changes in insulin sensitivity between lean and obese women during OC use.

Insulin resistance (ISI HOMA) seems to only worsen in obese women, not in lean women. Obesity is associated with chronic inflammation (23), which in turn plays a role in insulin resistance (24). Hormone replacement therapy may increase inflammation, as suggested by c-reactive protein concentrations, in postmenopausal women (25). However, whether exogenous hormones in the form of OCs induce inflammation in premenopausal women and whether obese women are particularly susceptible are unknown.

We also observed an increase in LDL levels by almost 20 mg/dL (a 16% to 20% increase) in both lean and obese women after 6 months of OC use. Most, but not all (26, 27) studies evaluating OCs containing cyproterone (28, 29), desogestrel (30), drospirenone (31), norethindrone (32), or levonorgestrel (33) did not report increases in LDL concentrations, even in women with disorders associated with insulin resistance such as polycystic ovary syndrome, gestational diabetes, or diabetes. One study reported an increase in midcycle LDL levels by 2.2% and 5.7% with 35 μg of ethinyl estradiol + norgestimate over 12 and 24 months (7). The timing of the LDL assessment may be important. Wiegratz et al. (34) evaluated lipid profiles on days 2, 11, and 21 at baseline and after 12 cycles of triphasic norgestimate and gestodene OCs. At the 12th cycle of OC use, the LDL concentrations were highest (36% above baseline) during the pill-free week, then lowered as the active pills commenced (10% above baseline). In our study, because women were assessed during their pill-free week, elevations of LDL by OC may have been magnified. However, elevations of LDL over baseline would be expected (34) even if LDL concentrations were evaluated at midcycle.

Our study had several limitations. First, we only evaluated metabolic parameters during OC administration for 6 months. Because contraception methods are most likely used for longer periods, future studies should evaluate the long-term effects of OCs in obese women. Second, we did not include a placebo group in the study, which would have been logistically difficult to perform. However, significant changes in metabolic parameters that were not due to the study medications would have been unlikely during a relatively brief 6-month period. Finally, a relatively small number of women were studied, so the lack of a statistically significant difference in some parameters (such as $AUC_{glucose}$) between the lean and obese women could have been due to a lack of power.

Lean and obese women seem to exhibit differential changes in insulin sensitivity when given 6 months of a commonly used OC. Both the mechanisms of these differences, such as chronic inflammation, and whether these divergent effects persist in the long term require further investigation.

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SUPPLEMENTAL MATERIALS AND METHODS

Laboratory Assays

Serum fasting total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride concentrations were measured by the central hospital laboratory. Other blood samples were centrifuged immediately, and the serum/plasma samples were stored at −70°C until assayed. Plasma glucose levels were determined using glucose oxidase methodology (YSI 2300 Stat Plus Glucose Analyzer; Yellow Springs Instruments), and the plasma insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (ALPCO Diagnostics). Serum fructosamine, an indicator of average glucose levels over the past 2 to 3 weeks, was analyzed via enzymatic assays (Glycated Serum Protein Enzymatic Assay, Diazyme Laboratories) by use of an automated clinical chemistry analyzer (Roche 911). All determinations were made in duplicate and averaged. All interassay and intra-assay coefficients of variation were <10%.

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Effect of Renin–Angiotensin System Inhibition on Cardiovascular Events in Older Hypertensive Patients with Metabolic Syndrome

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ABSTRACT

Objective. Metabolic syndrome (MetS) is associated with cardiovascular disease (CVD). Insulin resistance has been hypothesized as the underlying feature of MetS. Angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) are widely used antihypertensives that may improve insulin sensitivity. The aim of the study is to evaluate the effect of ACEI/ARB on incident CVD events in older hypertensive patients with MetS.

Materials/Methods. We used the Cardiovascular Health Study, a prospective cohort study of individuals > 65 years of age to evaluate ACEI/ARB use and time to CVD events (including coronary and cerebrovascular events). The study included 777 subjects who had hypertension and ATP III-defined MetS, but free of CVD and diabetes at baseline. Cox regression models were used to evaluate the effect of ACEI/ARB as compared to other antihypertensives on the time to the first CVD events.

Results. ACEI/ARB use was associated with a decreased risk of CVD events (adjusted HR = 0.658, 95 % C.I. [0.436–0.993]) compared to other antihypertensives. When CVD endpoints were evaluated separately, use of ACEI/ARB was associated with lower rates of angioplasty and coronary events (HR of 0.129 and 0.530 respectively, with 95 % CI [0.017–0.952] and [0.321–0.875]).

Conclusions. ACEI/ARB use was associated with a lower risk of CVD events in older hypertensive patients with MetS, primarily due to a reduction in coronary events. The potential protective effect of ACEI/ARB on CVD events in older individuals with MetS will need further confirmation from prospective studies.

Abbreviations: NCEP, National Cholesterol Education Program; ATP, Adult Treatment Panel; MetS, metabolic syndrome; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CHS, Cardiovascular Health Study; NHLBI, National Heart Lung and Blood Institute; MI, myocardial infarction; CHF, congestive heart failure; TIA, transient ischemic attack; CABG, coronary artery bypass graft; HR, hazard ratio; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; LIFE, Losartan Intervention for Endpoint Reduction; EUROPA, European Trial on Reduction of Cardiovascular Events with Perindopril in Stable Coronary Artery Disease; HOPE, Heart Outcomes Prevention Evaluation.

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1. Introduction

Metabolic syndrome (MetS), a constellation of metabolic risk factors, increases the risk for diabetes and cardiovascular events [1–3], including cardiovascular disease mortality [4–6], all-cause mortality [4–6], and coronary heart disease mortality [6]. The pathogenesis of MetS is complex and incompletely understood, but obesity and insulin resistance contribute to its development [7].

The National Cholesterol Education Program's (NCEP) Adult Treatment Panel III (ATP) criteria represent the most widely used definition for MetS. MetS, as defined by the ATP III criteria, is estimated to be prevalent in 28% of US adults [8]. The prevalence of MetS increases with age, reaching peak levels in the sixth decade for men and the seventh decade for women [9].

Current pharmacologic management of MetS focuses on the specific risk factors without targeting the underlying insulin resistance [10]. Several lines of evidence suggest that the renin–angiotensin system is both a contributor and target for several risk factors associated with the metabolic syndrome [11]. Angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) may improve insulin sensitivity [12–14], decrease the risk of type 2 diabetes [15], improve endothelial function [16], and reduce atherosclerosis and cardiovascular disease risk [17]. Whether ACEI and ARB improve clinical cardiovascular outcomes in hypertensive older patients with MetS is yet to be investigated. The purpose of this study is to evaluate the association between the use of ACEI/ARB and incident cardiovascular events in older adults with hypertension and MetS.

2. Methods

2.1. Data Source

We used data from the Cardiovascular Health Study (CHS), a community-based prospective cohort study conducted by the National Heart Lung and Blood Institute (NHLBI) in adults aged 65 and older, to evaluate risk factors for the development and progression of cardiovascular events. The purpose and design of CHS have been published previously [18]. Briefly, the CHS consisted of over 5800 participants randomly selected from Medicare eligibility lists in four U.S. communities in North Carolina, California, Pennsylvania and Maryland. Data collected included demographics, current medication use, blood pressure, medical history, lifestyle habits, anthropometric measures, fasting blood chemistry, echocardiography, electrocardiography and carotid ultrasonography. For each cardiovascular condition at baseline, data from self-report were confirmed using components of the baseline examination and a validation protocol that included review of medical records and confirmation by treating physicians [19]. University of Vermont's Central Blood Analysis Laboratory analyzed each participant's blood chemistry, which was drawn in the morning after an overnight fast. Further details on laboratory and blood sampling procedures, examinations, and quality assurance protocols have been published previously [18,20–22]. Subjects were followed with annual clinic visits and interim 6-month phone calls for a total of 11 years, followed by telephone follow-ups only from year 11 to 15. For this analysis, we used only the first 11 years of validated event data, as data for cardiovascular events after year 11 were obtained from telephone self report without validation from medical records. The CHS was approved by the University of Washington's Data Coordinating Center and the investigational review boards at all locations. Analysis of CHS data for the purpose of evaluating the association between ACEI/ARB use and incident cardiovascular events was approved by the Virginia Commonwealth University Institutional Review Board.

2.2. Inclusion and Exclusion criteria

The subjects included in the present analyses included individuals from CHS who had used any antihypertensive medication during the study. In addition, these subjects met the ATP III criteria for MetS [10]. We excluded subjects with baseline diabetes (defined as having a fasting blood glucose $\geq$ 126 mg/dl or a 2-h serum glucose $\geq$ 200 mg/dl upon an oral glucose tolerance test with 75 g glucose, or use of diabetes medications). Subjects with a history of cardiovascular events, including myocardial infarction (MI), congestive heart failure (CHF), coronary heart disease, claudication, stroke, transient ischemic attack (TIA), angina and arrhythmia, were also excluded. Individuals with prevalent cardiovascular disease and diabetes were excluded because they were already at risk for cardiovascular events regardless of the presence of MetS. We then classified the subjects based on their exposure to ACEI or ARB during the study. Hence, the exposed group was composed of individuals who had used ACEI/ARB alone or combined with other anti-hypertensives, and the control group represented those who took anti-hypertensives other than ACEI/ARB.

2.3. Endpoints

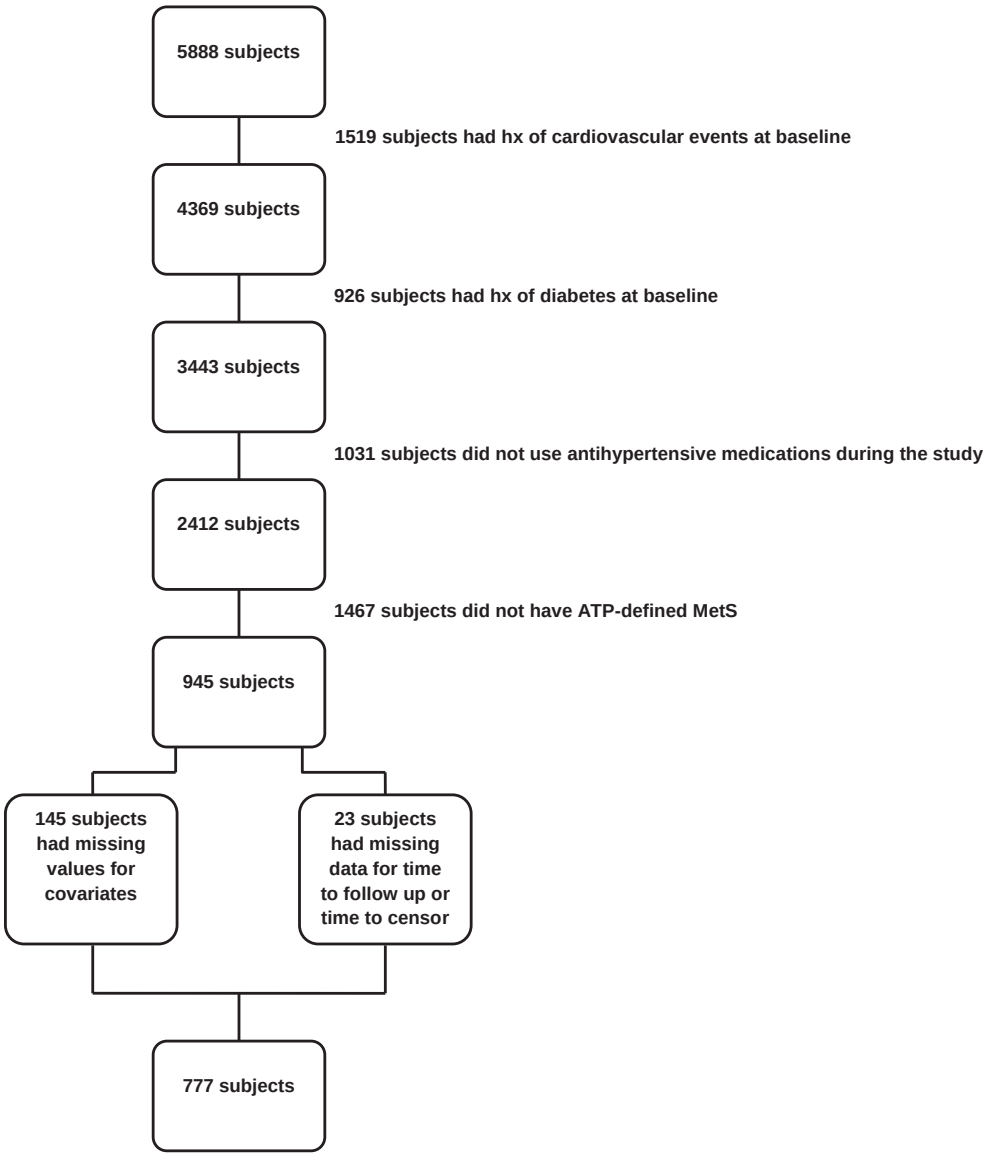
The primary endpoint was defined as the occurrence of *any* first cardiovascular event, including incident MI, silent MI documented by electrocardiogram, stroke, TIA, angioplasty, coronary artery bypass graft (CABG) procedures, angina, claudication, or death due to coronary heart disease during the 11 years of follow-up. The algorithms for identifying claudication [23], MI [24], stroke [21] and deaths due to coronary disease [24] have been reported previously. Secondary outcomes for this report included investigation of each of the following incident events separately: MI, silent MI, angina, CABG, angioplasty, claudication, stroke, TIA, as well as any coronary events and any cerebrovascular events. Coronary events included MI, CABG, angioplasty, angina, silent MI and deaths due to coronary disease. Cerebrovascular events included stroke and TIA.

2.4. Statistical analyses

A Cox hazards model with time dependent covariates was used to analyze the risk of developing cardiovascular events in users of ACEI/ARB compared to non-users, adjusting for potential confounders and possible significant interactions. Important risk factors for cardiovascular events were defined

a priori and were evaluated for inclusion in the multivariate model. These risk factors included age, cigarette use, family history of premature coronary heart disease, gender, alcohol use, exercise intensity as assessed by an instrument adapted from the Health Interview Survey [25], aspirin use, body mass index (BMI), LDL and HDL cholesterol levels, triglycerides, race, and income level. These were included as covariates in the multivariate model if their univariate *P*-values were <0.25, or as a confounder if its inclusion in the multivariate model changed the hazard ratio (HR) estimate by more than 20%. We also included systolic blood pressure and total number of antihypertensive medications used as time-dependent covariates in the preliminary models because blood pressure control itself is a risk factor for cardiovascular events. Development of diabetes and heart failure during follow-up were also included as time-dependent covariates to control for confounding by indications, as ACEI and ARB are commonly used in patients with heart failure and diabetes. Clinically plausible interactions were evaluated, including interactions between ACEI/ARB and age, ACEI/ARB and gender, and ACEI/ARB and race.

Preliminary multivariate models were compared using −2 log likelihood tests before a final multivariate model was constructed. The final model included the following covariates: development of diabetes, CHF, systolic blood pressure, age, gender (male vs. female), smoking status (current, former, never smoker), race (African American vs. other), triglycerides level, and LDL level. Subjects were censored if they did not develop any cardiovascular event during the follow-up period or if they left the study before the full follow-up. We assessed



ATP = Adult Treatment Panel; MetS = metabolic syndrome

Fig. 1 – Flow chart showing final sample size after applying inclusion/exclusion criteria.

the proportional hazard assumption and the goodness of fit of the multivariable model. P-values ≤ 0.05 was considered statistically significant.

3. Results

Of the original 5888 subjects enrolled in the CHS dataset, 1519 subjects had a history of cardiovascular events at baseline (including coronary heart disease, CHF, stroke or TIA). Of the remaining 4369 subjects, 3443 were free from diabetes at baseline. As we were interested in the subjects who used antihypertensive medications, we included in the analysis those who used at least one antihypertensive medication during the study (n = 2412). Out of these individuals, 945 had MetS as defined by ATP III at baseline. Twenty-three subjects had missing data for time to follow-up or time to censor, and 145 subjects had missing baseline values for covariates in the multivariable model. Our final sample size was 777 subjects as shown in Fig. 1. At baseline, 72 out of the 777 subjects (9.3%) were taking ACEI and none of the subjects at entry used ARB. The use of ACEI/ARB increased from baseline until year 11 where 26.1% used ACEI and/or ARB. The average duration of use of ACEI/ARB was 1.9 years (range 0 to 12 years).

Baseline characteristics of subjects were compared between those who took ACEI and/or ARB at baseline and the control group (Table 1). At baseline, there were no statistically significant differences between the 2 groups regarding their age, gender, smoking habits, triglycerides, HDL, LDL levels, BMI, total number of blood pressure medications used, fasting glucose or systolic blood pressure. However, the ACEI/ARB group contained a higher percentage of African Americans (21.0% in the exposed group vs. 11.1% in the control group, P = 0.0065). Rates of use of most antihypertensive medications (thiazide diuretics, potassium sparing diuretics, vasodilators and alpha blockers) were similar between the 2 groups. However, ACEI and/or ARB users were significantly less likely to use beta blockers (5.6% vs. 18.7%, P = 0.0051) but more likely to use loop diuretics (11.1% vs. 4.4%, P = 0.0129) or calcium channel blockers (18.1% vs. 8.7%, P = 0.0096) compared to the control group.

The percentage of subjects with uncontrolled blood pressure over the 11 years of follow up was compared between the ACEI/ARB and control group (Table 2). Over the follow-up period, blood pressure control was not significantly different between those who used ACEI/ARB and those who did not use any of these 2 classes of drugs except for year 3. In year 3, a higher percentage of subjects had uncontrolled blood pressure in the control group. To account for any possible difference in the control of blood pressure between the ACEI/ARB and control groups, systolic blood pressure was included in the model as time dependent variables. We used systolic blood pressure and not other measures of blood pressure in our analysis because systolic blood pressure has been more strongly associated with coronary heart disease than diastolic blood pressure. In addition, elevated systolic blood pressure is common among older individuals, which is our population under study [26,27].

Other important characteristics were also compared during the follow up years between the users of ACEI/ARB and non-users. There were minimal statistically significant differences regarding the percentage of subjects who developed CHF or diabetes over the 11 years of follow up between the ACEI/ARB group and the control group. However, to account for any possible differences, development of CHF and diabetes were assessed for inclusion in the multivariate model as time dependent variables (see Statistical Analyses).

Table 1 – Baseline comparison between subjects exposed to ACEI/ARB and the control group.

Covariate	ACEI/ARB users (N = 72)	Control group (N = 705)	P-value
Male	30 (42.0%)	230 (33.0%)	0.1214
Smoking			
Never	29 (40.0%)	360 (51.0%)	0.1937
Former	34 (47.0%)	262 (37.0%)	
Current	9 (13.0%)	83 (12.0%)	
Race			
White	56 (78.0%)	626 (88.8%)	
Black	15 (21.0%)	78 (11.1%)	0.0065
Other	1 (1.0%)	1 (0.1%)	
Triglycerides (mg/dl)	160.2 (±61.5)	165.4 (±63.9)	0.5109
HDL (mg/dl)	47.6 (±11.5)	49 (±12.6)	0.3785
LDL (mg/dl)	130.0 (±34.9)	136.2 (±35.0)	0.1502
Age (years)	71.8 (±4.4)	72.5 (±5.0)	0.2968
BMI (kg/m^2) [a]	28.7 (±3.8)	28.6 (±3.9)	0.9278
Number of antihypertensives	1.9 (±0.9)	1.7 (±0.8)	0.2074
Systolic blood pressure (mmHg)	143.8 (±21.4)	140.0 (±20.1)	0.2120
Fasting glucose (mg/dl)	105.3 (±7.5)	104.4 (±8.9)	0.3737
Frequencies of antihypertensive use at baseline			
Beta blockers	4 (5.6%)	132 (18.7%)	0.0051
Thiazides	7 (9.7%)	118 (16.7%)	0.1228
Loop diuretics	8 (11.1%)	31 (4.4%)	0.0129
K sparing diuretic	0 (0.0%)	10 (1.4%)	0.3091
Calcium channel blocker	13 (18.1%)	61 (8.7%)	0.0096
Vasodilators	10 (13.9%)	66 (9.4%)	0.2180
Alpha blockers	0 (0.0%)	28 (4.0%)	0.0850
Angiotensin receptor blocker	0 (0.0%)	0 (0.0%)	

Data are given as mean (SD) for continuous variables and as numbers (percent %) for categorical variables.
[a] BMI calculated as weight in kilograms divided by the square of height in meters.

The results of the univariate analyses in determining the risk of incident cardiovascular events are shown in Table 3.

The final model included exposure to ACEI/ARB, development of diabetes, CHF, systolic blood pressure, age, gender (male vs female), smoking status (current, former, never smoker), race (African–American vs other), triglycerides level, and LDL level. We tested for interactions between the use of ACEI/ARB and race, age, and gender; however, none of these interactions showed any statistically significant effects. The final multivariable model is presented in Table 4. In this final model, after adjusting for confounding variables, the use of ACEI or ARB was associated with a reduction in the risk of incident cardiovascular events (HR = 0.658, 95 % CI [0.436–0.993], P = 0.0462, Fig. 2).

We also assessed the effect of ACEI/ARB separately on coronary events (incident MI, silent MI, coronary heart disease death, CABG, angioplasty, or angina) and cerebrovascular

Table 2 – Prevalence of uncontrolled blood pressure (> 140/90 mmHg) in subjects exposed to ACEI/ARB and the control group over 11 years of follow-up.

Covariate	ACEI/ARB users (%)	Control group (%)	P-value
Baseline	56.94	62.37	0.3719
Year 1	95.56	92.91	0.5057
Year 2	88.64	92.41	0.3820
Year 3	38.16	55.84	0.0038
Year 4	47.47	56.50	0.0975
Year 5	54.87	58.93	0.4289
Year 6	49.55	59.64	0.0516
Year 7	51.24	60.16	0.0739
Year 8	53.15	61.15	0.0881
Year 9	56.58	64.47	0.0817
Year 10	50.00	56.09	0.1800
Year 11	58.24	61.79	0.4265

Table 4 – Multivariate model for the risk of incident cardiovascular events in ACEI/ARB users vs. non-users.

Parameter	Hazard Ratio (HR)	95% HR Confidence Limits		P-value
Use of ACEI/ARB	0.658	0.436	0.993	0.0462
Development of CHF	7.566	5.312	10.775	<.0001
Systolic blood pressure (mmHg)	1.007	1.001	1.013	0.0151
Development of diabetes	1.419	0.618	3.256	0.4093
Age (years)	1.035	1.010	1.061	0.0065
Gender (male vs. female)	2.140	1.643	2.788	<.0001
Former smoker vs. never	1.218	0.919	1.615	0.1707
Current smoker vs. never	2.142	1.486	3.088	<.0001
Race (black vs. non-black)	0.808	0.488	1.339	0.4090
Triglycerides (mg/dl)	1.003	1.001	1.005	0.0050
LDL (mg/dl)	1.004	1.001	1.008	0.0153

events (incident stroke and/or TIA). The use of ACEI/ARB had a significant protective effect against the development of coronary events (HR = 0.530, 95 % CI [0.321–0.875], P = 0.0130). In particular, when angioplasties were evaluated alone, the ACEI/ARB exposure decreased risk of first angioplasty (HR = 0.129, 95% CI [0.017–0.952], P = 0.0446). However, there were no effects on cerebrovascular events (HR = 1.173, 95% CI [0.621–2.217], P = 0.6228). These data suggest that the effects of ACEI/ARB may differ between coronary and non-coronary cardiovascular events.

Additionally, we also evaluated the effect of ACEI/ARB on all-cause mortality. The use of ACEI/ARB did not have a significant effect on all-cause mortality in both univariate (HR =1.068, 95% C.I. [0.713–1.600], P = 0.7494) and multivariate models (HR = 1.078, 95% C.I. [0.714–1.629], P = 0.7198).

Table 3 – Univariate analyses of potential risk factors, including exposure to ACEI/ARB, for incident cardiovascular events.

Variable	Hazard Ratio (HR)	95% HR Confidence Limits		P-value
Age (years)	1.052	1.027	1.077	<.0001
Gender (male vs. female)	2.039	1.593	2.611	<.0001
Smoking (former vs. never)	1.363	1.041	1.785	0.0242
Smoking (current vs. never)	2.149	1.509	3.062	<.0001
Race (black vs. non-black)	0.621	0.379	1.019	0.0594
Number of alcohol beverages/week	1.017	1.000	1.033	0.0460
Aspirin use (user vs. non-user)	1.174	0.906	1.521	0.2246
Exercise intensity level (absent, low, moderate, high)	1.124	0.958	1.319	0.1506
BMI (kg/m^2)	0.989	0.957	1.021	0.4817
Income level [a]	1.030	0.965	1.098	0.3769
Family history of myocardial infarction	1.013	0.778	1.319	0.9230
Triglycerides (mg/dl)	1.002	1.000	1.004	0.0400
HDL (mg/dl)	0.986	0.976	0.997	0.0122
LDL (mg/dl)	1.001	0.998	1.005	0.4481
Use of ACEI/ARB	0.782	0.523	1.168	0.2292
Systolic blood pressure (mmHg)	1.008	1.002	1.014	0.0064
Development of diabetes	1.030	0.454	2.335	0.9439
Development of CHF	7.122	5.094	9.958	<.0001
Number of antihypertensives	0.922	0.820	1.038	0.1785

[a] Income level is divided into 8 categories: under $5000, ($5000–$7999), ($8000–$11,999), ($12,000–$15,999), ($16,000–$24,999), ($25,000–$34,999), ($35,000–$49,999), (>$50,000).

3.1. *Sensitivity analyses*

We performed sensitivity analyses by including waist circumference and insulin sensitivity in the multivariate model because they are closely related to the metabolic syndrome. Waist circumference did not have a significant association with incident cardiovascular events in univariate (HR = 1.010, 95% CI [0.998–1.022]) or multivariate (HR = 1.007, 95 % C.I. [0.995–1.020]) models. Inclusion of waist circumference in the multivariate Cox model shows that the hazard ratio for incident cardiovascular events was 0.656 in ACEI/ARB users vs. control (95% C.I. [0.435–0.991], P = 0.0452), which is similar to the estimates without the inclusion of waist circumference. Similarly, insulin sensitivity (by homeostasis model assessment [HOMA]) did not have a significant association with incident cardiovascular events in both univariate (HR = 0.953, 95% CI [0.902–1.008]) or multivariate (HR = 0.953, 95 % C.I. [0.899–1.010]) analyses. Inclusion of HOMA in the multivariate Cox regression model shows that the hazard ratio for incident cardiovascular events in ACEI/ARB users was 0.660 as compared to the control group (95% CI [0.437–0.996], P = 0.0476), which is similar to the estimate obtained without the inclusion of HOMA as a covariate. Because waist circumference and HOMA were not independently associated with risk of incident cardiovascular events, and their inclusion did not result in a change in the original estimates, the final model did not include waist circumference or HOMA.

To validate the conclusions obtained from the multivariable model, we also tested the effect of using ACEI/ARB on incident cardiovascular events after further adjusting for the concurrent use of other antihypertensives by including them as time dependent variables in the models (Table 5). These

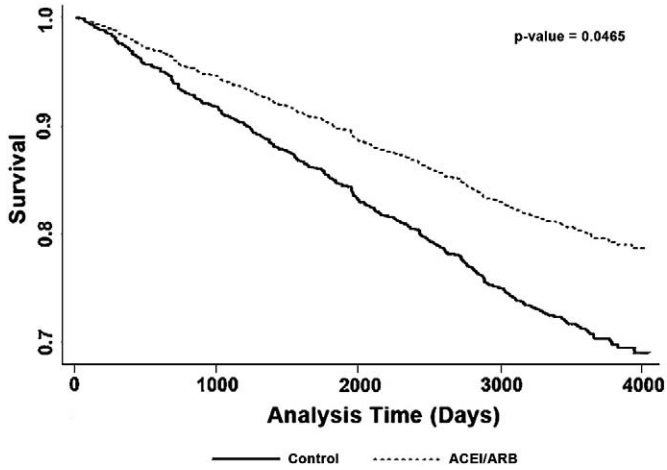


Fig. 2 – Cox regression survival plot comparing the survival rate (free from incident cardiovascular events) in subjects exposed to ACEI/ARB as compared to the control group.

models also allowed us to evaluate the effect of each of the following classes of anti-hypertensives: beta blockers, alpha blockers, calcium channel blockers, diuretics and vasodilators on the outcomes. Concurrent use of these other anti-hypertensive classes did not change the magnitude of association between ACEI/ARB and the incidence of cardiovascular events. We found that the use of ACEI/ARB, independent of concurrent use of other antihypertensive class of drugs, was associated with a significant protective effect against the development of cardiovascular events (HR = 0.644, 95 % CI [0.426–0.976], $P = 0.0379$).

Table 5 – Effect of different antihypertensives on incident cardiovascular events.

Parameter	Hazard Ratio (HR)	95% HR Confidence limits		P-value
Use of ACEI/ARB	0.644	0.426	0.976	0.0379
Use of beta blockers	0.864	0.609	1.226	0.4130
Use of CCB	0.920	0.653	1.296	0.6323
Use of vasodilators	0.854	0.428	1.704	0.6548
Use of diuretics	0.965	0.737	1.263	0.7968
Use of alpha blockers	0.881	0.359	2.158	0.7811
Development of CHF	7.547	5.279	10.791	<.0001
SBP	1.008	1.002	1.014	0.0103
Development of diabetes	1.429	0.623	3.282	0.3994
Age	1.034	1.009	1.06	0.0087
Gender (male vs. female)	2.142	1.642	2.795	<.0001
Former smoker vs. never	1.209	0.911	1.604	0.1886
Current smoker vs. never	2.124	1.473	3.062	<.0001
Race (black vs. other)	0.826	0.497	1.373	0.4610
Triglycerides	1.003	1.001	1.005	0.0046
LDL	1.004	1.001	1.008	0.0185

4. Discussion

MetS is highly prevalent in older individuals [9] and has been associated with future cardiovascular events [1–3]. ACEIs and ARBs may have beneficial effects on insulin sensitivity [12–14], the major underlying pathophysiologic feature of MetS. Few studies reported the effect of ACEI/ARB in patients with MetS [28–30]. These studies were short-term, and the effects of ACEI/ARB on the clinical cardiovascular endpoints were not assessed. Therefore, we sought to determine whether ACEI/ARB inhibition prevents cardiovascular events in hypertensive older patients with MetS, after excluding those with diabetes and any history of cardiovascular disease at baseline.

In this study, we observed a lower risk of incident cardiovascular events among older hypertensive individuals with MetS who used ACEI/ARB (adjusted HR = 0.658, 95% CI [0.436–0.993], $P = 0.0462$]). Our findings complement those of large randomized controlled trials such as HOPE and EUROPA [17,31], which supported the use of ACEI for secondary prevention of cardiovascular events. HOPE showed a significant reduction in the rate of death, MI, stroke, revascularization, cardiac arrest, and heart failure with the use of an ACEI (ramipril) in patients at high risk for cardiovascular events (age > 55 years old with preexisting coronary disease or equivalent, with at least one other risk factor such as smoking, hypertension, or dyslipidemia). Similarly, EUROPA showed that an ACEI (perindopril) reduced the primary endpoint of cardiovascular death, MI or cardiac arrest in a population with stable coronary heart disease. Both EUROPA and HOPE evaluated a population at higher risk of cardiovascular events (individuals with preexisting coronary heart disease) than our current evaluation (individuals with hypertension and MetS but without diabetes or preexisting coronary disease). To our knowledge, the current study is the first to evaluate this lower-risk population.

The Cox regression survival curves in Fig. 2 for individuals taking ACEI/ARB and those using other anti-hypertensives start to separate at one to two years after the start of the follow-up period, and the difference in incident

cardiovascular events (survival curves) between the 2 groups increases with time. Although survival benefits seen within such a relatively short time frame may seem like a chance finding, our results are congruent with the findings of HOPE, where Kaplan–Meier curves for the ramipril and placebo groups started to separate between the first and second years of follow up, and continued to diverge during the 4.5 years of follow-up. In the HOPE Study Extension (HOPE-TOO), cardiovascular benefits of ramipril were maintained during an additional 2.6 years of post-trial follow-up, regardless of baseline risk or other concomitant treatments [32]. This suggests that treatment with ramipril in HOPE may have prolonged beneficial effects. The survival curves in Fig. 2 corroborate findings in HOPE-TOO, and suggest that exposure to ACEI/ARBs may have extended benefits. However, this will need to be further evaluated in future studies.

When coronary and cerebrovascular events were evaluated separately, we found a significant reduction in risk of coronary events (MI, silent MI, coronary heart disease death, CABG, angioplasty, or angina) with the use of ACEI/ARB (adjusted HR = 0.530, 95% CI [0.321–0.875], $P = 0.013$]). However, the risk of developing cerebrovascular events was not different between users and non-users of ACEI/ARB. This suggests that ACE/ARB's effects might be different between coronary and non-coronary cardiovascular events.

One possible reason that ACEI/ARB do not have beneficial effects on cerebrovascular accidents in this study is that MetS according to the ATP-III definition may not be a strong predictor of stroke and/or TIA risk [33–35]. This may explain the lack of effect in our study consisting only of individuals with MetS based on the ATP-III definition. Another reason may be that regardless of the presence of MetS, ACEI/ARB may not be protective against the risk of cerebrovascular events, as was previously reported [36]. Sub-analysis by race in the LIFE study, for example, showed that African–American participants treated with losartan were actually at higher risk for stroke events than African–American individuals who received atenolol [36]. The analysis of several double-blinded randomized controlled trials has also suggested that the use of ACEI might not be protective against stroke and may be associated with greater risk for stroke [37].

Although it is encouraging that ACEI/ARB may be protective against cardiovascular events, and especially coronary events, in this cohort of older individuals with hypertension and MetS, we should interpret our findings in light of the strengths and weaknesses of the study. The strengths of our study include the long duration of follow-up, reliable recording of cardiovascular events, prospective documentation of cardiovascular risk factors and medication assessments, which occurred at baseline and at annual follow-ups. Study limitations include the relatively small sample size after applying the inclusion and exclusion criteria on the total number of participants. In addition, residual confounding may be present. For example, there may be unmeasured systematic differences between patients prescribed ACEI/ARB and those who were not. It is possible that the healthcare providers selected ACEI or ARB for patients who were at increased risk of developing cardiovascular disease, such as patients with risk of developing diabetes and heart failure. Although we have adjusted for the development of diabetes and heart failure in our final model by including them as time-dependent variables to control for confounding by indication bias, it is still possible that participants who received ACEI/ARB may have received a different level of care as compared to participants who did not. In addition, the proportion of subjects taking ACEI/ARBs is small, which reflects the practice pattern at the time of observation. Despite the smaller sample, use of ACEI/ARBs was significantly associated with a lower incidence of cardiovascular events, even after adjusting for other risk factors. Another limitation is that some patients may have changed their lifestyle (smoking habits, exercise intensity, number of alcohol beverages consumed per week) during the study and this could not be accounted for in the analysis. Finally, the results of this study may not be generalizable to patients younger than 65 years as only older subjects were included in CHS.

Our results are translatable to the care of older patients with hypertension and MetS. ACEI/ARBs are the preferred treatment for blood pressure control in patients with diabetes [38]. Whether this class of drug is also first-line for hypertensive patients with MetS is unknown. To the authors' knowledge, this current report is the first to address this knowledge gap. Pending validation from prospective clinical trials, ACEI/ARBs may become the preferred treatment for hypertension management in patients with MetS.

In summary, the results of the present study show that use of ARBs or ACEIs may be associated with decreased risk of cardiovascular events, particularly coronary events, in hypertensive older subjects with MetS. Our results require validation from prospective clinical trials.

Author Contributions

H.H.Z. collected and analyzed data, and drafted the manuscript. K.I.C. designed the study, obtained funding, supervised data collection and analysis, and finalized the manuscript. S.E.H., D.P.M. and P.W.S. contributed to data interpretation and analyses.

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Acknowledgments

None.

Conflict of interest

There is no relevant conflict of interest to be disclosed.

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1. SPECIFIC AIMS. African Americans have more morbidity and mortality from cardiovascular disease and diabetes than Caucasians. Moreover, these disparities are especially prominent in women. Insulin resistance plays a pathophysiologic role in both disorders. Even when adjusted for BMI and other factors, African Americans remain more insulin resistant than Caucasians. The oral contraceptive (OC) is the most commonly used contraceptive method, and OC may affect insulin sensitivity. Indeed, our preliminary data suggest that OCs worsen insulin sensitivity especially in women who have underlying insulin resistance, and this effect is, in part, mediated by changes in estrogen metabolism (**3.2**). Thus, African-American women, who are more insulin resistant, may be at risk of aggravating that insulin resistance with OC use. However, little data exist on OCs' effects on insulin sensitivity and cardiovascular risk in African-American women. This knowledge gap is a critical problem, because OCs are often used long-term, sometimes for decades, and small increases in cardiovascular risk may have important clinical consequences.

The PI's *long-term goal* is to elucidate the role of hormones on insulin resistance and cardiovascular risk in women. The *objective* of this application is to determine the effect of OCs on insulin sensitivity and cardiovascular risk in African-American versus Caucasian women, and the role of estrogen metabolites in mediating these differences. **We hypothesize that OC use will impair insulin sensitivity and worsen the cardiovascular risk profile more so in African-American women as compared with Caucasian women, and that differential estrogen metabolism in part mediates this racial difference.** Our hypothesis has been formulated based on our preliminary data (**3.1-3.3**) and supported by evidence from other groups demonstrating that: (i) insulin sensitivity worsens with OC use, especially in women with already impaired insulin sensitivity; (ii) OC administration alters endogenous estrogen metabolism; and (iii) the estrogen metabolites, 2-hydroxyestradiol and 2-hydroxyestrone, regulate glucose homeostasis. We will test our hypothesis by pursuing the following specific aims:

Specific Aim 1: Determine the estrogen metabolism profile in African-American and Caucasian women before and during OC use. Evolving data suggest that estrogen metabolites mediate cardiovascular and metabolic risk; however, human data regarding estrogen metabolism have been limited due to the availability of accurate assays. In this aim, we will measure estrogen metabolites using state-of-the-art high performance liquid chromatography-tandem mass spectrometry. Our *working* hypothesis is that estrogen metabolite profiles differ between African-American and Caucasian women before and during OC use.

Specific Aim 2: Determine the extent to which insulin sensitivity and cardiovascular risk profile differ between African-American as compared with Caucasian women, before and during OC administration. Based on our preliminary data and observations from others, OCs may worsen insulin sensitivity, flow-mediated dilatation (FMD) and carotid intima media thickness (CIMT) in some women, especially those who have underlying insulin resistance. However, whether these effects of OCs differ between African-American women and Caucasian women is unknown. Our *working hypothesis* is that OC use will result in a more pronounced worsening in (i) insulin sensitivity, (ii) FMD, and (iii) CIMT in African-American women.

Specific Aim 3: Determine the relationship between OC-associated alteration in estrogen metabolism and changes in insulin sensitivity and cardiovascular risk profile in African-American as compared with Caucasian women. Our preliminary data suggest that the degree of insulin sensitivity deterioration during OC use is related to changes in estrogen metabolism (**3.2**). In this aim, we will evaluate whether these changes in estrogen metabolism during OC use are also accompanied by worsening in FMD and CIMT, in addition to the expected changes in insulin sensitivity. We will also determine whether racial differences exist.

Expected outcomes: (1) To our knowledge, this will be the first study to *prospectively* determine racial differences in insulin sensitivity and cardiovascular risk with OC use. (2) The role of estrogen metabolites in these metabolic and cardiovascular risks will be clarified. (3) Results will inform future investigations on the role of estrogen metabolites as biomarkers to tailor contraceptive choices to specific patients. (4) Future studies can also elucidate whether

polymorphisms in estrogen metabolizing enzymes affect cardiovascular risk. Thus, important advances in the prevention of metabolic and cardiovascular consequences would be expected.

2. BACKGROUND AND SIGNIFICANCE

2.1. Significance: African Americans have increased cardiovascular morbidity and mortality, including myocardial infarction, stroke, and hypertension (1). These disparities are especially prominent in women under 65 years of age. In this group, African-American women have twice the number coronary deaths and diabetes (a coronary risk equivalent) as Caucasian women (2-4). Insulin resistance is a precursor to diabetes and plays a pathophysiological role in coronary atherosclerosis (5;6). Our preliminary data, as well as data from others, suggest that African-American women may likely be at increased risks of developing insulin resistance, and therefore cardiovascular risk, when using OCs. However, appropriate prospective studies are lacking. Mechanisms for worsening insulin sensitivity with OC administration are also poorly understood. Lack of such knowledge is a critical problem, because the clinical use of OC with the least harm will not be possible until its metabolic and cardiovascular risks are clearly defined. Our contribution with the proposed studies is the detailed comparison of insulin sensitivity, FMD and CIMT before and during OC use between African-American and Caucasian women, and to evaluate the role of estrogen metabolites in mediating these differences. ***This contribution is significant because of its immediate and long-term expected outcomes. (1) Results from the proposed studies will immediately inform the risks versus benefits of OC use, such that contraceptive choices can be further tailored based on an individual's metabolic and cardiovascular risk profile. (2) This contribution is also the first step in a continuum of research towards understanding the physiology of estrogen metabolites in insulin resistance and cardiovascular risk*.** Once these physiological processes are understood, factors that influence hormonal regulation of insulin resistance and cardiovascular risk can be studied. For example, as estrogen metabolism is controlled by cytochrome (CYP) P450 and catechol-O-methyl transferase (COMT) enzymes (7-9), strategies may be developed to regulate the intricacies of the estrogen metabolic pathways. Also, since these metabolizing enzymes are highly polymorphic (10), results of this research may also have cardiovascular implications for individuals with specific metabolizing enzyme genetic polymorphisms. Furthermore, the findings of the proposed studies will likely be applicable to postmenopausal women, in whom the cardiovascular risk associated with the use of hormone replacement is highly debated. Thus, important advances in the prevention of cardiovascular diseases and disorders associated with insulin resistance would be expected.

2.2. Innovation: The racial disparity in cardiovascular disease and diabetes between African Americans and Caucasians have been well documented, and this disparity is especially prominent in women 35-64 years of age (4). However, the physiological processes underlying this racial difference (11) are seldom studied and poorly understood. The limited studies in this area have mostly evaluated the role of obesity (12) or socioeconomic status (13) in African Americans, and tend to employ cross-sectional observations (14). ***The research proposed in this application focuses on a novel approach, the role of exogenous estrogen and their associated changes in endogenous estrogen metabolism on racial differences in insulin sensitivity and cardiovascular risk factors.*** Additionally, as discussed in **4.1.4**, we will quantitatively measure estrogen metabolites using high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS^2), in collaboration with Dr. Veenstra at the National Cancer Institute (see letter of collaboration). **This innovative quantification method is expected to overcome the poor specificity and reproducibility from enzyme immunoassays** (15;16). Our interdisciplinary team of investigators consists of a clinical pharmacotherapist experienced in insulin sensitivity modeling, three cardiologists and a mass spectrometry expert. Our approach will yield findings that expand the understanding of the biologic mechanisms contributing to insulin resistance, and subsequently, cardiovascular risk in women.

2.3 Background

2.3.1. Racial Disparities in Coronary Disease and Insulin Resistance. The racial disparities in myocardial infarction, stroke and hypertension have been well established (1;4). Among women in particular, African Americans suffer from coronary deaths (4) and diabetes (2;3) at younger ages (**Fig 2.3.1**), and at twice the rate of Caucasians. Although social factors and higher rates of obesity may contribute to these health disparities, endothelial dysfunction (17) and insulin resistance (18;19) have been demonstrated even in African-American adolescents and pre-pubertal children, before dietary and environmental effects are expected. In addition, even when matched for body mass index (BMI), African Americans remain more insulin resistant as compared with Caucasians (12). These evidence suggest that biological processes, rather than just social/ environmental factors alone, are at least in part responsible for the increased risk of coronary heart disease (CHD) and diabetes in African Americans.

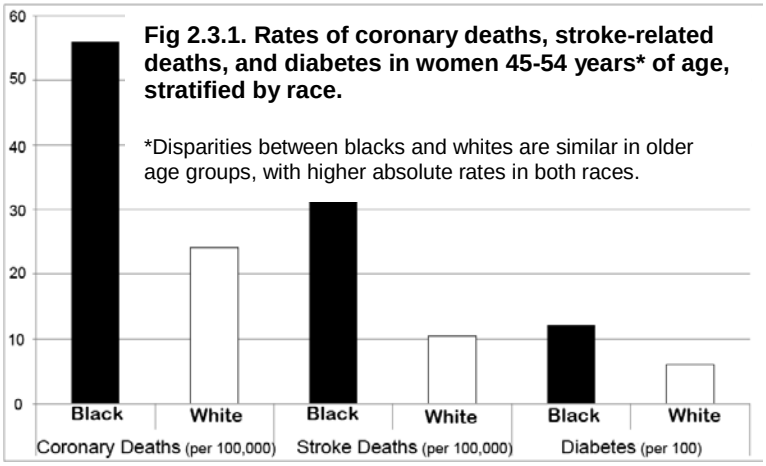


Fig 2.3.1. Rates of coronary deaths, stroke-related deaths, and diabetes in women 45-54 years* of age, stratified by race.

*Disparities between blacks and whites are similar in older age groups, with higher absolute rates in both races.

2.3.3. Role of Insulin Resistance in Cardiovascular Events. Current epidemiological data strongly suggest insulin resistance is an independent risk factor for CHD. Insulin resistance (6) and hyperinsulinemia (20), with (21) or without (22) impaired glucose tolerance, independently predicts risk of CHD, severity of CHD, and CHD mortality. Additionally, insulin resistance is significantly associated with CHD risk even after adjusting for traditional risk factors (such as age, sex, lipids, cigarette smoking and blood pressure) and the metabolic syndrome (5). Individuals with the lowest insulin sensitivity have the highest risk of CHD (6). Moreover, in African-American adolescents, the degree of endothelial dysfunction is directly correlated with the degree of hyperinsulinemia (17).

Numerous studies have investigated the biological mechanisms of insulin resistance on atherosclerosis. Individuals with insulin resistance demonstrate decreased endothelial nitric oxide production (23) and impaired microvascular dilatation capacity (24). Coronary blood flow response to cold pressor test is reduced in insulin resistant patients (25), and this defect is reversible by insulin sensitizing drugs (25). Numerous investigations have also demonstrated that systemic inflammation and insulin resistance coexist (26).

2.3.4. Oral Contraceptives, Insulin Resistance and Cardiovascular Disease. In African-American and Caucasian women, the hormonal combined oral contraceptive (OC) pill is the most commonly used contraceptive method (27). Among American women, 82% use OCs sometime in their lifetime (27). In fact, 4 of the 200 most commonly prescribed medications in the U.S. are hormonal OCs containing ethinyl estradiol and a progestin (28).

OCs may impair insulin sensitivity in some women (29-32). In *low risk, healthy, normal-weight* women, OCs containing ≤35 µg ethinyl estradiol have limited effects on insulin sensitivity (33). However, our preliminary data revealed that in women with impaired insulin sensitivity (but without overt diabetes or pre-diabetes), OCs further deteriorated insulin sensitivity (see **3.1**). Hence, African-American women, who already have impaired insulin sensitivity compared with Caucasian women (12), may be at risk of aggravating that insulin sensitivity when they use OCs. However, little information exists regarding the effect of OCs on insulin sensitivity in African-American women. In the only study available, African-American women who were using OCs had increased insulin resistance, glucose intolerance, and serum triglycerides, as compared to African-American women who were not using OCs (33). This study was cross-

sectional in nature, and although suggestive of increased pre-diabetes and cardiovascular risk with OC use (33), limited conclusions could be drawn.

We also previously reported meta-analysis data indicating a 2-fold increase in risk of myocardial infarctions and ischemic strokes among current low-dose OC users as compared with non-users or prior-users (34). Our data are congruent with epidemiological observations (35). OCs also increase thromboembolic risk and hypertension (36).

If OC use increases cardiovascular events in women without underlying risk factors, women who already have metabolic dysfunction prior to starting OCs would be even more susceptible to adverse cardiovascular effects. Indeed, OC use worsens endothelial function and carotid intima media thickness (CIMT) in hyperandrogenic women with impaired insulin sensitivity (37).

2.3.5. Estrogen Metabolism as Modulator of Insulin Sensitivity. Endogenous estrogen metabolism plays a role in regulating glucose metabolism, and may explain OC's deleterious effects on insulin sensitivity. Endogenous estradiol is predominantly oxidized via two major competing pathways (oxidation at C-2 yielding 2-OH estrogens such as 2-hydroxyestradiol [2OHE2] and 2-hydroxyestrone [2OHE1], vs. oxidation at C-16 yielding 16α-OHE1) (38) (**Fig. 2.3.5**). Oxidized metabolites can further be methylated by COMT enzymes. These estrogen metabolites possess various physiological properties (39).

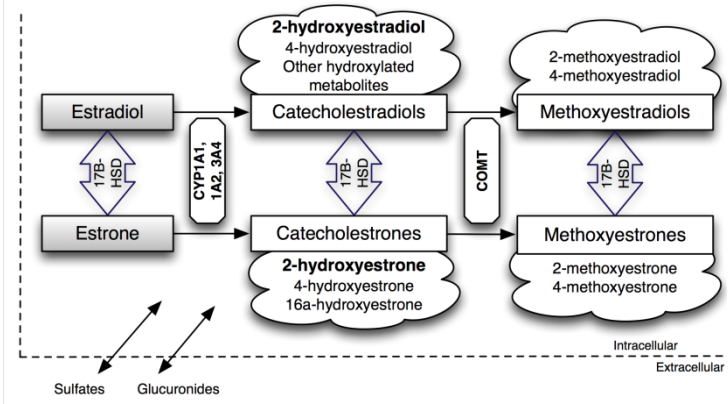


Fig. 2.3.5. Multiple mechanisms for estradiol metabolism. Cytochrome P-450 (CYP) and Catechol-O-metholtransferase (COMT) enzymes are important enzymes determining the exact composition of estrogen metabolites. Multiple lines of evidence suggest that the estrogen metabolites, **2-hydroxyestradiol and 2-hydroxyestrone** modulate insulin sensitivity and prevent metabolic complications of insulin resistance.

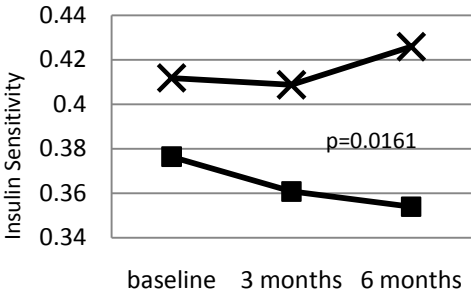
17B-HSD=17β-hydroxysteroid dehydrogenase; Dotted line represents cellular membrane.

Several lines of evidence suggest that the estrogen metabolites 2OHE2 and 2OHE1 regulate glucose homeostasis. 2OHE2 mediates intracellular signaling of AMP-activated protein kinase (40), which leads to reduction in fat synthesis and improved glucose uptake (41). 2OHE2 administration in obese rats also attenuated obesity, blood pressure and dyslipidemia, and improved endothelial function as compared with control treatment (42). In humans, low urinary 2OHE1 concentrations were correlated with elevated serum insulin, indicative of insulin resistance, in women with the polycystic ovary syndrome (43). Lastly, in non-diabetic women, we have demonstrated that an increase in plasma concentrations of 2OHE2 over time predicts improvement in insulin sensitivity (see **3.2**). In summary, cumulative evidence suggests that 2-OH estrogens mediate processes that govern insulin sensitivity. Importantly, our preliminary data suggests that administration of OCs alters endogenous estradiol metabolism (**3.2**). OC use *decreases* 2-OH estrogens, and shifts the metabolic pathway towards other estrogen metabolites (44), which may explain development of insulin resistance in some women using OCs.

2.3.6. Gap in Knowledge. To summarize, although beginning evidence suggest that African-American women may be at an increased risk of developing insulin resistance and the accompanying cardiovascular risk when using OCs as compared with Caucasian women, no appropriate prospective studies have been done. Lack of such knowledge is a critical problem. Until the risks of OCs are clearly defined, the clinical use of this common pharmacologic agent with the least harm would not be possible.

3. PRELIMINARY STUDIES

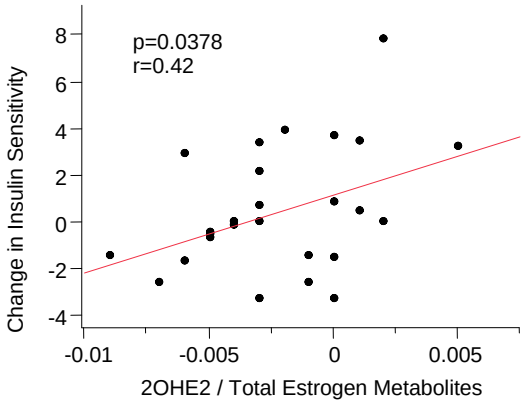
Figure 3.1. Further Deterioration in Insulin Sensitivity by Oral Contraceptive Use in Women Who Had Underlying Impaired Insulin Sensitivity



Women with different baseline insulin sensitivity used OC (ethinyl estradiol 35 mcg and norgestimate 0.18/0.215/0.25 mg) for 6 months. Insulin sensitivity was measured at baseline, 3 months and 6 months using frequent sampling IV glucose tolerance tests (FSIVGTT), and other indices (QUICKI, ISI-HOMA, and Matsuda index). Women with lower insulin sensitivity at baseline (■) experienced further deterioration of insulin sensitivity while on OC. Women with higher insulin sensitivity at baseline (×) did not have worsening of insulin sensitivity during OC use. This effect was seen regardless of the method used to measure insulin sensitivity (p=0.0494, p=0.0161, p=0.0128, p=0.0227, for FSIVGTT, QUICKI, ISI-HOMA, and Matsuda index, respectively), suggesting that the results were internally consistent.

Using available epidemiological data, the degree of impairment in insulin sensitivity imparted by OC use translates to an increase in risk of CHD by 4% (45). Additionally, following OC administration, fructosamine (glycated albumin) which represents mean glucose over the previous 2 weeks, increased from 270 to 320 μmol/L (equivalent to an increase of 40 mg/dL in mean glucose) significantly in women who had underlying insulin resistance. Although a mix of Caucasian and African-American women were studied, they were not directly comparable because they were not BMI-matched at baseline. We propose to compare BMI-matched African-American and Caucasian women in the proposed studies.

Figure 3.2. Oral Contraceptive Use Alters Endogenous Estradiol Metabolism, and Changes in 2-OH Estrogen Metabolites are Associated with Changes in Insulin Sensitivity



Women were administered OC and followed for 6 months. Estrogen metabolites and insulin sensitivity were evaluated at baseline and 6 months. Concentrations of 2OHE1 and 2OHE2 were measured using LC-ESI-MS^2, and expressed as a fraction of total estrogen metabolites, and as a ratio to16α-OHE1. We observed that OC use for 6 months *significantly reduced* plasma concentrations of (i) 2OHE2 (p=0.05), 2OHE2/16α-OHE1 (p<0.001), and 2OHE2/total estrogens and estrogen metabolites (p=0.002); (ii) 2OHE1/16α-OHE1 (p=0.001) and 2OHE1/total estrogens and estrogen metabolites (p=0.003).

In addition, change in insulin sensitivity as measured by FSIVGTT was significantly predicted by changes plasma concentrations of 2OHE2, expressed as a ratio of 2OHE2 to total estrogen metabolites. These data suggest that metabolic **shifts toward** 2OHE2 (↑ in 2OHE2/ Total estrogen metabolites) may be favorable to insulin sensitivity, while metabolic **shifts away** from 2OHE2 (↓ in 2OHE2/ Total estrogen metabolites) are associated with insulin resistance.

Figure 3.3. Reversal of Insulin Resistance Improves Flow-Mediated Dilatation

We have also previously evaluated whether improving insulin sensitivity with metformin improves FMD of the brachial artery in hyperandrogenic women with the polycystic ovary syndrome during 3 months of OC administration. When insulin sensitivity improves with the use of metformin, FMD improves as well.

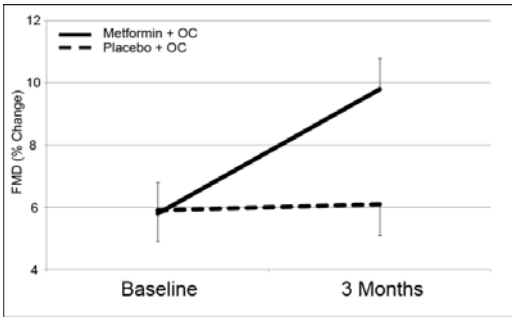


Table 3.4. Tertiles of Insulin Sensitivity and CIMT in Non-diabetic Elderly > 65 years of age (46)

	Insulin Sensitivity Tertile		
	Lowest (n=8)	**Middle (n=7)**	**Highest (n=8)**
Si	2.1 ± 0.54	3.9 ± 0.45	7.5 ± 2.5
Total CIMT	6.8 ± 0.5	7.7 ± 0.2	7.4 ± 1.1
Plaque Score	14.3 ± 8.7	10.2 ± 7.5	0.9 ± 1.8
Plaque Number	7.6 ± 4.6	4.0 ± 3	0.5 ± 1.0

In an elderly population, we have evaluated insulin sensitivity (S_i) using FSIVGTT, along with CIMT, assessing: (i) total CIMT (sum of CIMT measurements from 6 pre-specified sites), (ii) number of plaques, and (iii) plaque score (sum of maximal height of each plaque) (46). Atherosclerosis severity is directly associated with severity of insulin resistance. Although these data were not derived from the target study population, which are premenopausal females desiring OCs, they provide credence that worsening insulin sensitivity worsens CIMT, and that CIMT assessments proposed are feasible.

3.5. Feasibility of Proposed Studies. The PI, together with the multi-disciplinary team of investigators, is uniquely posed to conduct the series of studies outlined in this application. As evidenced by our preliminary data, we have extensive experience in accurately measuring: (i) insulin sensitivity using FSIVGTT; (ii) estrogen metabolites

using state-of-the-art LC-ESI-MS^2, (iii) FMD and CIMT using established methods. We have also demonstrated successful recruitment of both African-American and Caucasian women for contraceptive studies. Hence, our demonstrated experience provides assurance that the proposed studies will be successfully completed in the timeframe allowed by this award.

4. DESIGN AND METHODS

We hypothesize that OC use will impair insulin sensitivity and worsen the cardiovascular risk profile more so in African-American women as compared with Caucasian women, and that differential estrogen metabolism in part mediates this racial difference. We will test our hypothesis by pursuing the 3 Specific Aims, which will be addressed by a single project, as outlined below.

4.1. Study design. We will prospectively evaluate insulin sensitivity, FMD and CIMT in 33 African-American and 33 Caucasian women, at baseline, 3 and 6 months, during administration of a commonly used OC (ethinyl estradiol 35 mcg and norgestimate 0.25 mg, Ortho Cyclen®, Ortho-McNeil Pharmaceutical). This OC preparation is chosen because it is one of the most commonly prescribed (28). Our preliminary data were also obtained using an OC preparation with the same estrogen and progestin. The study will take place at Virginia Commonwealth University (VCU) Clinical Research Center (CRC). Participants will be evaluated as described in **Table 4.1.**

Table 4.1. Protocol

Visit	Study Procedures
Pre-study	Informed consent, anthropometric measures (height, weight, waist and hip circumference), vital signs, history, labs (CBC and SMA20) to determine eligibility.
Baseline	**Day 1:** CIMT and FMD prior to OGTT. Procedures performed after a 12-hr fast. Measurement of anthropometric variables. 24-hour urine collection. Plasma for fasting lipid panel, hsCRP, sex steroids (estrogen, estrone, estradiol) and estrogen metabolites. 2-hr OGTT with glucose & insulin determinations every 15 min. Fast after 2000 h. **Day 2:** Procedures performed after a 12-hr fast. FSIVGTT to determine insulin sensitivity, pancreatic insulin secretion and glucose action. Begin OC administration after FSIVGTT.
Phone	Monthly phone monitoring of side effects and compliance.
Week 12	Repeat Day 1 procedures (CIMT, FMD, anthropometric measures, 24-hour urine collection, fasting lipid panel, hsCRP, sex steroids, estrogen metabolites, 2-hr OGTT).
Phone	Monthly phone monitoring of side effects and compliance.
Week 24	**Day 1:** Repeat Baseline Day 1 procedures (CIMT, FMD, anthropometric measures, 24-hour urine collection, fasting lipid panel, hsCRP, sex steroids, estrogen metabolites, 2-hr OGTT). **Day 2:** Repeat Baseline Day 2 procedures (FSIVGTT to determine insulin sensitivity, pancreatic insulin secretion and glucose action).

OGTT = oral glucose tolerance test; FSIVGTT = Frequent sampling intravenous glucose tolerance test

4.1.1. Entrance Criteria. Inclusion criteria: 1) premenopausal, regular-cycling women 18-35 years; 2) either African-American or Caucasian; 3) non-smoker. African-American and Caucasian women will be BMI-matched. Exclusion criteria: 1) Diabetes by A1c, fasting glucose or a 2-hour oral glucose tolerance test (OGTT); 2) clinically significant pulmonary, cardiac, renal, hepatic, neurologic, psychiatric, infectious, and malignant disease; 3) contraindications to OC use (history of thromboembolism, acute coronary or cerebrovascular ischemic events, vascular disease, coagulopathy, prolonged immobilization, breast cancer, migraine head-aches, major surgery within past 6 months, blood pressure >160/100 mmHg, pregnancy or lactation); 4) use of OCs or other hormonal contraceptives, glucose-lowering medications, anti-hyperlipidemic, anti-hypertensive or other vasoactive drugs within previous 3 months.

4.1.2. CRC Visits and Study Procedures.

4.1.2.1. Day 1—CIMT, FMD and OGTT studies. On Day 1, all women will be in the follicular phase of the menstrual cycle, as documented by serum progesterone concentration < 2 ng/mL. The women will present to the CRC after a 12-hour overnight fast, and their weight, height, waist-to-hip ratio, and blood pressure will be measured. They will undergo OGTT, FMD (to

assess endothelial function), and CIMT (to assess progression of atherosclerosis). To prevent potential discomfort during phlebotomy from exerting vasoactive effects on vascular imaging, we will perform CIMT and FMD before OGTT. All vascular images will be captured on videotape by a single experienced ultrasonographer and digitalized for later analysis by a single experienced cardiologist. Intra-observer variations will be established. Image processing software has been developed by the CRC bioengineer to streamline this process.

Carotid Ultrasonography. B-mode ultrasound imaging of the right and left carotid arteries will be performed with Philips ATL 5000 (Phillips Medical Systems, Andover, MA) ultrasound equipped with a 5-12 MHz broadband linear array transducer. Patients will be in the supine position with the head turned 45° contralateral to the side of the scanning. Mean common carotid artery IMT on the posterior wall 5-10 mm from bifurcation will be obtained. The common carotid, versus other segments, is studied because the measurement yield and reproducibility is higher than those from other segments (47). We will define IMT as average distances between the far wall lumen-intima and media-adventitia ultrasound interfaces, bilaterally, taken at end-diastole. B-mode and color Doppler examinations will be performed in both longitudinal and transverse planes to identify stenoses to take into consideration circumferential asymmetry. We will define plaques as focal intrusions into the lumen $\geq$ 1.0 mm (48), considering the age, sex and race of participants being studied. IMT will be measured from digitalized images using electronic calipers by a single board certified experienced cardiologist, Dr. Lotun (see letter of collaboration). Given our study population, we do not anticipate severe atherosclerosis. However, should we find severe plaques, individuals will be referred to one of the cardiologists in this study for further risk stratification.

Flow-mediated Dilatation Studies. After CIMT measurements, endothelial function will be assessed by brachial artery FMD. After a rest period of 15 minutes, a sphygmomanometer cuff will be positioned on the upper right forearm, and a broadband (7-12MHz) linear array ultrasound transducer will be placed longitudinally on the upper arm, 5-7cm above the antecubital crease, to obtain baseline brachial artery images and flows, along with heart rate. A section of the vessel will be chosen that is a fixed distance from an anatomic marker such as a fascial plane or vein in cross-section. This will allow the same section of the vessel to be re-imaged on the subsequent follow-up visits. The forearm cuff will be inflated to a pressure of 300mmHg for 4.5 minutes, and then deflated abruptly, resulting in a marked increase in brachial artery blood flow. Blood flow velocity will be recorded for 15 seconds after cuff deflation, and the brachial artery will be imaged during the next 1.5 minutes to record changes in vessel diameter during the increased flow induced by hyperemia (endothelium-dependent vasodilatation). After a 15-minute rest period to allow for vessel recovery, baseline images will again be recorded. Subsequently, sublingual nitroglycerine (NTG) spray (400µg) will be administered and 3 minutes later, imaging will be performed again to determine endothelial-independent vasodilatation. Brachial artery diameter will be measured using a high-resolution electronic caliper system. Three diameter measurements will be averaged for baseline recordings, and 4 post-hyperemia diameter measurements will be assessed between 55-60 seconds after cuff deflation for all subjects. Diameter measurement will be made at end diastole coincident with the ECG-R wave and will be made between the near wall and far wall m-line on the vessel. Similar measurement techniques will be used for the NTG images. Response variables will be the percent vessel dilation during hyperemia (FMD). All images will be evaluated by a single board certified experienced cardiologist, Dr. Arrowood (see letter).

Oral Glucose Tolerance Test and Blood Sampling. After a rest period of 30 min (>5 half-lives of the NTG given during FMD studies), 3 sets of baseline blood samples will be drawn 10 minutes apart for measurements of plasma concentrations of insulin, glucose, and estrogen metabolites. Fasting lipid panel, and hsCRP will be obtained. These cardiovascular markers are assessed since they are commonly measured clinically, and are also expected to follow the direction of change in insulin sensitivity. Subsequently, 75g of glucose will be given orally

to each woman, and blood samples will be collected every 15 min for the next 2 hours for determination of glucose and insulin concentrations. Instructions on 24-hr urine collection will be given. Although the FSIVGTT (Day 2) will give the most accurate assessment of insulin sensitivity, we also perform OGTT on Day 1, as the OGTT can be replicated clinically. Hence, worsening of glucose tolerance detected by OGTT can be used as a clinical monitoring parameter.

4.1.2.2. Day 2—Frequently Sampled IV Glucose Tolerance Test. On Day 2, after a 12-hour overnight fast, the subjects will have insulin sensitivity measured using FSIVGTT (49-51). At time 0, 300mg/kg dextrose IV bolus will be administered over 1 minute, and 0.03units/kg insulin will be administered similarly 20 minutes later. Blood samples will be assayed for glucose and insulin at 0, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160, 180 minutes as specified by the Bergman minimal model method (49-51). The dynamic responses of insulin and glucose during IV glucose tolerance testing will be analyzed by sophisticated computer modeling (MINIMOD®) to yield determinations of insulin action (tissue insulin sensitivity, S_i), glucose action (insulin-independent glucose disposal, Sg), acute insulin response to glucose (AIRg, adequacy of insulin secretion), and disposition index (DI, or AIRg x S_i, a composite of insulin secretion and action). The PI has extensive experience with the FSIVGTT technique (52;53).

The "gold standard" for measuring insulin sensitivity has been the insulin-glucose clamp. However, this technique is highly labor intensive and complex, and limits the number of women who could realistically be evaluated. In contrast, the FSIVGTT is optimal for screening a larger number of subjects, as in our case. Several studies have demonstrated that insulin sensitivity measurements obtained by the FSIVGTT correlate excellently with those obtained by the insulin-glucose clamp (54-56). Additionally, our group has demonstrated that intra-subject variation in measurements of insulin sensitivity were minimal when FSIVGTT in healthy males were measured on three separate occasions separated from each other by one month (57).

After FSIVGTT, participants will be educated on the administration of OC study medication, and provided a supply of OC (ethinyl estradiol 35 mcg and norgestimate 0.25 mg). The OC will be taken as one tablet daily for 21 days per month followed by a 7-day pill-free period.

4.1.2.3. Follow Up. Women will be phoned monthly to assess adverse effects and compliance. After 12 weeks, during the first week of active pills of the fourth OC pack, the subjects will return to the CRC after a 12-hour overnight fast and will undergo the same measurements performed on Day 1. At week 24, during the first week of active pills of the seventh OC pack, women will return to the CRC and repeat day 1 and 2 of study procedures (**Table 4.1**).

4.1.3. Laboratory Assays. Serum fasting total, LDL, and HDL cholesterol, triglyceride, progesterone and hsCRP concentrations will be measured via routine hospital laboratory tests. Other blood samples will be centrifuged immediately, and sera and plasma samples stored at –70°C until assayed. Plasma glucose will be determined using glucose oxidase methodology (YSI 2300 Glucose Analyzer, Yellow Springs, OH), and plasma insulin levels will be measured via ELISA (ALPCO Diagnostics, Salem, NH), with inter- and intra-assay coefficients of variation (CV) of 5.4-8.6% and 2.9-6.2%, respectively. All analytes will be assayed in duplicate.

4.1.4. Quantification of Estrogen Metabolites. Estrogen metabolites will be quantified via high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (58;59) (HPLC-ESI-MS^2) by Dr. Veenstra at National Cancer Institute (see letter of collaboration). This method quantifies 15 estrogens and estrogen metabolites simultaneously, including: 2OHE1 and 2OHE2, which are the metabolites of interest in this aim, and estrone, estradiol, estriol, 2-methoxyestradiol, 2-methoxyestrone, 2-hydroxyestrone-3-methyl ether, 4-hydroxyestrone, 4-methoxyestrone, 4-methoxyestradiol, 16α-hydroxyestrone, 17-epiestriol, 16-ketoestradiol, and 16-epiestriol. The measurements have high sensitivity (limit of detection of 2.5 fmol/ml plasma), precision (CV's $\leq$ 5%), specificity and accuracy. The assay also

measures total estrogen metabolites (sum of glucuronidated, sulfated and unconjugated metabolites). This quantification method is expected to overcome the poor specificity and reproducibility from commercially available immunoassays (15;16).

4.1.5. Recruitment. Participants will be recruited from VCU student health clinics, VCUHS obstetric/gynecology clinics, via posters displayed around VCU campuses, through newspaper advertisements, health magazines, websites, city and campus buses, and Richmond Planned Parenthood. We have regularly recruited women volunteers successfully from these venues.

4.1.6. Sample Size. We estimated our sample size with both the primary metabolic outcome, insulin sensitivity (S_i), and the primary cardiovascular outcome, FMD. FMD, instead of CIMT, is used for sample size estimation because endothelial dysfunction precedes atherosclerosis.

We used our preliminary data to estimate sample size for the primary metabolic endpoint, S_i as measured by FSIVGTT. We have shown that women with underlying insulin resistance will experience further reduction in S_i (-1 min^{-1}/mu/L) after using OC for 6 months, while women with normal S_i at baseline will have S_i improved slightly (+0.2 min^{-1}/mu/L). We used these figures to calculate our sample size to detect a racial difference, based on numerous reports indicating that African Americans have decreased insulin sensitivity (12;18;19) when compared with their Caucasian counterparts. Assuming a common standard deviation of 1.3 min^{-1}/mu/L, we will need to study 20 African-American women and 20 Caucasian women to achieve a power of 0.8 with 2-sided α=0.05.

To estimate sample size for the primary cardiovascular outcome, FMD, we used existing literature suggesting that FMD decreases from 8.7% to 6.4% (a reduction of 2.3%) in South American (predominantly black or mixed race) OC users (60), while it remains unchanged in normal-weight Turkish women (37). Assuming FMD will decrease by 2.3% in African-American women, vs. 0.75% in Caucasian women, following 6 months of OCs, with a common standard deviation of 2.0%, we will need 28 women in each group, with 80% power and 2-sided α=0.05.

Hence, the sample size calculation for the entire project (Aims 1-3) is based on the calculations performed for FMD, since the largest number of subjects is needed for this endpoint. Attrition during this 6-month study is expected to be 15%, based on PI's previous clinical studies involving OCs in this population (61). Hence we will recruit 33 African-American women and 33 Caucasian women for the entire project.

4.2 Specific Aim 1: Determine the estrogen metabolism profile in African-American and Caucasian women before and during OC use

4.2.1. Rationale. Evolving data suggest that estrogen metabolites mediate cardiovascular and metabolic risk; however, human data regarding estrogen metabolism have been limited due to the availability of accurate assays. In this aim, we will measure estrogen metabolites using state-of-the-art HPLC-ESI-MS^2. Our *working hypothesis* is that estrogen metabolite profiles differ between African-American and Caucasian women before and during OC use.

4.2.2. Study Design, Entrance Criteria, CRC procedures, Recruitment and Sample Size have been described in sections **4.1** through **4.1.6**.

4.2.3. Data Analysis. 2OHE1 and 2OHE2 will be assessed alone, as a fraction of total estrogens and metabolites, and as a ratio to 16α-OHE1, yielding i) 2OHE1, ii) 2OHE2, iii) 2OHE1/total estrogens and metabolites, iv) 2OHE2/total estrogens and metabolites, v) 2OHE1/16α-OHE1, and vi) 2OHE1/16α-OHE1, in plasma and urine (per mg urinary creatinine). Expressing values as a fraction to total estrogens and as a ratio to 16α-OHE1 will reflect shifts in estrogen metabolic pathways, since oxidation at the C-2 and the C-16 positions involve mutually exclusive pathways (38). Although not our primary outcome parameters, urinary estrogen metabolites will also be evaluated to ascertain internal consistency of our data. Normality will be examined with the Wilk-Shapiro test. Results not normally distributed will be log-transformed. African-American women will be compared to Caucasian women at

baseline by the 2-tailed students' *t* test. Change within each group over 6 months of OC use will be compared by the 2-tailed paired *t*-test. Differential change in the estrogen metabolites of interest between the two groups over the 6 months of OC will be evaluated by repeated measures analysis of variance (ANOVA), testing for interaction between the estrogen metabolites' time-trends and race status. $P<0.05$ will be considered significant.

4.2.4. Interpretation of Results. Population distribution data of estrogen metabolites quantified with accurate assays do not currently exist. Because African-American women are more insulin resistant than Caucasian women, and that lower 2OHE2 and 2OHE1 levels are associated with insulin resistance, we expect: (1) African-American women will have (i) lower plasma concentrations of 2OHE1 and 2OHE2, and (ii) lower ratios of 2OHE1/total estrogens and metabolites, 2OHE2/total estrogens and metabolites, 2OHE1/16α-OHE1, and 2OHE1/16α-OHE1. (2) African-American women will also have a more dramatic reduction in the above estrogen metabolites during OC use as compared with Caucasian women.

4.2.5. Potential Pitfalls and Alternatives. Our hypothesis for Aim 1 is that lower concentrations of 2-OH estrogens at baseline and/or a more dramatic reduction in these estrogen metabolites during OC use will be observed in African-American vs. Caucasian women. Although our preliminary data (**Fig. 3.1-3.2**), and data on insulin resistance in African-Americans (12;18;19), support this hypothesis, it is possible the estrogen metabolite profile may not be different between the 2 groups. If this should occur, we will first examine other estrogen metabolites, with particular attention to 2-methoxyestradiol, as it has been implicated in cardiovascular function (62-64). Even if we encounter negative results, we would have provided the first published evidence using *accurate quantification methods*, that estrogen metabolites do not differ between these 2 races. Other biological reasons for racial differences in insulin sensitivity can be pursued. Importantly, OC's effects on insulin resistance, FMD and CIMT (Aim 2), and relationships between changes in estrogen metabolites and changes in these parameters (Aim 3) are not dependent on results from Aim 1.

4.3 Specific Aim 2: Determine the extent to which insulin sensitivity and cardiovascular risk profile differ between African-American as compared with Caucasian women, before and during OC administration

4.3.1 Rationale. Based on our preliminary data and observations from others, OCs may worsen insulin sensitivity, FMD and CIMT in some women, especially those who have underlying insulin resistance. However, whether these effects of OCs differ between African American women and Caucasian women is unknown. Our *working hypothesis* is that OC use will result in a more pronounced worsening in (i) insulin sensitivity, (ii) FMD, and (iii) CIMT in African-American women.

4.3.2. Study Design, Entrance Criteria, CRC procedures, Recruitment and Sample Size have been described in sections **4.1** through **4.1.6**.

4.3.3. Data Analysis. The outcomes of interest are (i) Insulin sensitivity (S_i, from FSIVGTT), (ii) FMD, and (iii) CIMT. Other glucose metabolism variables to be analyzed are fasting insulin and glucose, areas-under-the-curve for insulin and glucose during OGTT; and AIRg, Sg and DI during FSIVGTT (see **4.1.2.2**). Results not normally distributed will be log-transformed. Differential changes in (i) S_i, (ii) FMD, (iii) CIMT, and (iv) other glucose metabolism parameters between African-American and Caucasian women during OC use will be evaluated by repeated measures ANOVA, testing for interaction between the time-trends of these parameters and race status. We will also determine the relative time course of changes in these parameters using mixed-model repeated-measures ANOVA. $P<0.05$ is considered significant.

4.3.4. Interpretation of Results. We expect that S_i will decline further in African-American women as compared with Caucasian women following OC administration. FMD will diminish, and CIMT will increase more so in African-America women, following a deterioration in S_i.

4.3.5. Potential Pitfalls and Alternatives. One drawback is that FMD and CIMT are surrogate endpoints of cardiovascular risk. Although using surrogate endpoints usually represent a limitation, an event-driven study is currently unforeseeable without prior data supporting a differential racial effects upon OC use. Our proposed study would provide such data. Another drawback is that CIMT and FMD assessments cannot be blinded due to the absence of a placebo group. A placebo-controlled study involving contraception is impractical and unethical given the risk of the pregnancies in the placebo group. Another potential pitfall is that the study period may be insufficient to detect significant changes in FMD and CIMT. This is unlikely as numerous studies have detected differences in these variables in the same 6 months of study period (37;60;65) in at-risk women. However, should we observe no significant changes in FMD and CIMT, we will examine the time course of alteration in all parameters to inform the design of future studies with longer intervention periods. Additionally, although our preliminary data (**3.1**) support our hypothesis that insulin sensitivity will deteriorate more markedly in African-American women, there is a possibility that African-American and Caucasian women may exhibit the same insulin sensitivity profiles when using OCs. One purpose of this study is to evaluate for and document potential racial differences if they occur. Should our hypothesis be not supported by our data, we would have provided the first published *prospective* evidence that commercially available OCs do not worsen insulin sensitivity more so in African-American women. Such results will advance the field, because the only other available study in the area, using cross-sectional data, suggests worsened metabolic risk in African-American OC users (33). Without ascertainment of baseline differences in metabolic profiles, limited conclusions regarding racial differences could be drawn from this prior study. Also, should we encounter a lack of difference between the 2 groups, we will evaluate changes in estrogen metabolites alongside with changes in insulin sensitivity (Aim 3) to explain this lack of difference.

4.4 Specific Aim 3: Determine the relationship between OC-associated alteration in estrogen metabolism and changes in insulin sensitivity and cardiovascular risk profile in African-American as compared with Caucasian women

4.4.1 Rationale. Based on our preliminary data, the degree of insulin sensitivity deterioration during OC use is associated with alteration in estrogen metabolism (**3.2**). In this aim, we will evaluate associations between alteration in estrogen metabolism and changes in (i) insulin sensitivity, (ii) FMD, and (iii) CIMT during OC use, and whether racial differences exist.

4.4.2. Study Design, Entrance Criteria, CRC procedures, Recruitment and Sample Size have been described in sections **4.1** through **4.1.6**.

4.4.3. Data Analysis. The outcomes of interest are changes (Δ, from baseline to 6 months) in the estrogen metabolites of interest and changes in S_i, FMD and CIMT. The main independent variables are: (i) Δ2OHE1, (ii) Δ2OHE2, (iii) Δ2OHE1/total estrogens and metabolites, (iv) Δ2OHE2/total estrogens and metabolites, (v) Δ2OHE1/16α-OHE1, and (vi) Δ2OHE1/16α-OHE1. The primary dependent variables are: (i) ΔSi, (ii) ΔFMD, and (iii) ΔCIMT. Normality of changes will be evaluated. For normally distributed data, Pearson's correlations will be performed to evaluate relationships between changes in independent and dependent variables. For nonparametric data, Spearman's correlations will be performed. African-American and Caucasian women will be evaluated separately, and together with race as an interaction term to evaluate whether race modulates estrogen metabolites' metabolic and cardiovascular effects. $P<0.05$ will be considered significant.

4.4.4. Interpretation of Results. We expect that decrease in 2OHE1 and 2OHE2, expressed alone, as a ratio to 16α-OHE1, or as a fraction of total estrogens and metabolites, will be associated with (i) deterioration in S_i, (ii) reduction in FMD, and (iii) increase in CIMT.

4.4.5. Potential Pitfalls and Alternatives. Our hypothesis for Aim 3 is that a deterioration of S_i, FMD and CIMT will accompany a reduction in the 2OH estrogen metabolites as a result of OC use. Although it is possible that OC administration may not lead to a reduction in 2OH

estrogen metabolites, this is highly unlikely as both our preliminary data (**3.2**) and data from others (44) support that OC use *decreases* 2OH estrogens, and shifts the metabolic pathway towards other estrogen metabolites. Another potential pitfall is that although our preliminary data support that a reduction in 2OH estrogens with OC use is associated with diminished Si, this may not translate to a deterioration in FMD or CIMT in a study period of 6 months. Although this scenario is possible, it is unlikely as numerous studies (37;60;65) and our preliminary data (**3.3**) have detected changes in these variables in as little as 3 months.

4.5. Timeline. We will simultaneously study all aims.

Month	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
Team meetings among PI, collaborating cardiologists, CRC staff & ultrasonographer	■								
Recruit participants and conduct protocol for Aims 1, 2 & 3		■	■	■	■	■	■	■	
Analyze and report results									■

4.6. Future Directions. Our *long-term goal* is to elucidate the role of hormones on insulin resistance and cardiovascular risk in women. At the conclusion of the proposed studies, we would have developed data supporting or refuting racial differences in insulin resistance and cardiovascular risk profile in women taking OCs. If the findings of this study support such a racial difference, the next logical step will be to examine if other baseline risk characterization, e.g. estrogen metabolites, obesity, hsCRP, predict the magnitude of the racial difference. In addition, other commonly used OCs can be studied. The estrogen metabolites important in mediating insulin sensitivity and cardiovascular risk profile identified in this study will inform future study designs to evaluate methods to regulate the intricacies of the estrogen metabolism pathways. Additionally, the enzymes for estrogen metabolism (**Fig. 2.3.5**) are highly polymorphic (10). Results of this research will inform the design of future pharmacogenomic studies in identifying proper candidates for OCs or estrogen replacement, such that use of exogenous estrogens with the least harm will be possible. Our data will also inform future investigations on estrogen metabolites as biomarkers of cardiovascular risk. Thus, important advances in the prevention of metabolic and cardiovascular consequences would be expected.

5. Human Subjects. (1) Subjects: Only women will be studied because OCs are exclusively used in women. No vulnerable populations (e.g. pregnant women, children, prisoners, or institutionalized individuals) will be studied. (2) Potential risks: Common side effects with OCs include nausea/vomiting, abdominal discomfort, breast tenderness, edema, and weight changes. Rare but serious adverse events (AEs) include thromboembolism, stroke, severe hypertension, and myocardial infarction. As noted in **4.1.1**, individuals at risk for these severe AEs will not be studied. Participants will also be monitored monthly by phone. Other potential risks are pain and infection at phlebotomy sites. Standard precautions to maintain sterility will be used. Hypoglycemia could occur during FSIVGTT. Participants will be closely monitored by the PI and medical staff. During FMD procedures, potential discomfort may occur when the blood pressure cuff is inflated to 300 mmHg. Participants may voluntarily withdraw from the study at any time. (3) Adequacy of protection against risk: Approval from the VCU IRB and CRC board will be obtained. The PI will obtain written informed consent from all potential participants prior to study procedures. The consent form will include a description of the study in layman's terms, potential risks/benefits, alternative procedures (in this case, the alternative is to choose not to participate), and investigators' and IRB's contact information. Potential participants will be given ample time, without coercion, to read the consent document and decide whether to participate. Study records will be held under strict confidentiality. Other than study numbers, no identifying information will accompany samples or electronic image files. (4) Potential benefits: There are no immediate benefits to the participants. The risks are minimal. The data to be obtained will increase the participant's knowledge of her cardiovascular risk. (5) Data safety and monitoring: The study team will evaluate protocol compliance and risks every 3 months. Additionally, periodic review by the CRC Research Subject Advocate, who functions as an independent compliance officer, will occur every 6 months to 1year. All AEs will be reported to the IRB and CRC Advisory Committee within 48 hours.

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RESEARCH STRATEGY

1. Significance

The polycystic ovary syndrome (PCOS) is a prevalent disorder that affects approximately 6-10% of women of childbearing age (1;2). PCOS is the most common cause of anovulatory infertility in women. The economic burden of PCOS is significant—$500 million is spent annually in the U.S. on treating PCOS-associated infertility alone (3). About 50% to 70% of women with PCOS are obese (4;5), and weight reduction ameliorates hyperandrogenemia, insulin resistance and menstrual irregularity associated with this disorder (6-8). Weight loss can also lead to resumption of menses and spontaneous pregnancies (6;9-11). Hence, in obese PCOS women, lifestyle intervention can be an initial or adjunctive treatment in the management of reproductive and metabolic dysfunction (12). However, adherence to lifestyle modification programs is notoriously poor, even under the close monitoring within randomized control trials (13). Research on effective lifestyle advice for infertile patients is lacking and inadequate to guide clinical practice (14). Without effective interventions, weight loss as an initial or adjunctive treatment strategy for PCOS-related infertility is highly impractical.

Motivational interviewing (MI) is a directive, patient-centered counseling approach that helps patients explore and resolve ambivalence about behavior change (15). An important principle of MI is that motivation for change must come from the individual rather than a health care "expert." Thus the interaction is collaborative and conducive, not coercive to change. Historically, health care providers share information regarding the consequences of unhealthy behaviors to persuade change within an expert/recipient paradigm; however these strategies may actually increase resistance to change (15;16). MI has shown efficacy in facilitating adoption of health behaviors in substance abuse (17), and in pediatric (18;19) and adult (20-24) weight loss. However, patients attempting to conceive may have different intrinsic motivation than these general populations (25), and the efficacy of MI in facilitating weight loss in infertile women with PCOS has not been evaluated. *Our proposed research will provide* a detailed understanding of the relative effectiveness of MI compared with conventional lifestyle counseling in obese women with PCOS who desire pregnancy, and the moderators / mediators for weight loss treatment effect. *This contribution is significant because it is the first step toward a continuum of research in developing a clinically translatable approach to weight loss in obese infertile women with PCOS.* Once we have established MI's efficacy in weight loss in PCOS, we can evaluate how MI can be directly integrated into the clinical care of these women. This research will also contribute to a broader understanding of the moderators and mediators of weight loss treatment effect, which can inform future trial designs to evaluate more targeted weight loss interventions. With effective strategies, weight loss intervention can be more readily used as an initial or adjunctive management of PCOS-related infertility in clinical practice. Results of our proposed studies will also form the basis for future investigations on the effect of adoption of health behaviors on psychological well-being and pregnancy rates (26). Obesity is not only associated with anovulation (27;28). It decreases fertility even in ovulatory women (29;30), and has serious adverse effects on both the fetus and mother (31;32). Thus, findings from this study might be applicable to preconception care.

2. Innovation

Although many investigators have demonstrated that weight loss of 5–10% reduces hyperandrogenism (33), improves menstrual cyclicity (34) and fertility (35) in obese women with PCOS, adherence to lifestyle modification programs is poor with high attrition rates (12). Hoeger et al. reported a 39% drop-out rate in a controlled trial of intensive lifestyle modification and/or metformin therapy in overweight women with PCOS (13). Also, such an intensive program with an exercise physiologist, a dietician, weekly or biweekly meetings is not practical for translation into clinical practice. Baillargeon et al. evaluated simple weight loss advice delivered in the clinic to obese women with PCOS, but one-third of the participants dropped out within 6 months (36). *This proposed research is innovative because no studies to date have evaluated a patient-centered weight loss approach in women with PCOS that takes into consideration patients' values and baseline readiness*. MI is such an approach. Our preliminary studies (see **3.1.2.1**) suggest that MI will be effective in facilitating weight loss. This new and substantively different technique is a departure from traditional weight loss interventions and is expected to overcome high attrition rates, and the practical problems in translating standard weight loss programs into clinical practice. We expect that effective weight loss therapy for overweight PCOS women will lead to improved reproductive function and fertility. This will also be the first study to evaluate moderators and mediators of weight loss treatment effect in women with PCOS.

3. Approach

We hypothesize that in obese women with PCOS desiring pregnancy, motivational interviewing (MI) will enhance weight loss effectiveness as compared to a conventional counseling approach. We propose to test our hypothesis via the following 2 Specific Aims, which will be addressed by a single project.

3.1 Specific Aim 1: Compare the effect of motivational interviewing (MI) vs. traditional lifestyle counseling on (i) weight loss, (ii) adoption of health behaviors, (iii) menstrual cyclicity, and (iv) psychological well-being, in overweight and obese women with PCOS who desire pregnancy

3.1.1. Rationale. Although weight loss can be beneficial to the reproductive function of women with PCOS, adherence to weight loss advice is low. Obese women with PCOS may present with a wide range of readiness to begin lifestyle changes. MI is a patient-centered, empathetic counseling style developed to help patients work through ambivalence and commit to change. The *objective* of this aim is to evaluate the effectiveness of MI intervention in facilitating weight loss in overweight/ obese women with PCOS. Our *working hypothesis* is that MI is superior to conventional lifestyle counseling in effecting weight loss in obese women with PCOS, and successful weight reduction will lead to improved menstrual cyclicity and psychological well-being. In this aim, we will randomize women with PCOS to MI vs. traditional lifestyle counseling and assess changes from baseline to 3, 6, and 12 months in (i) weight, (ii) health behaviors, (iii) menstrual cyclicity, and (iv) psychological well-being. Results of this aim will inform the development of more effective weight loss strategies for overweight/obese women with PCOS, and other obese non-PCOS women attempting to conceive.

3.1.2. Preliminary Studies

3.1.2.1 Feasibility of MI Intervention. Dr. Bean, co-investigator and a clinical health psychologist, has developed an MI program to encourage health behaviors in obese adolescents. Preliminary data suggest participants receiving MI had higher gym compliance (80.3% vs. 78.3%), nutrition compliance (88.0% vs. 78.6%), and remained in the weight loss program longer as compared to a control group not receiving MI. Fidelity to the MI approach was also maintained throughout the program (Table 1). *These data support feasibility for the MI intervention as proposed in Aim 1, and support the hypothesis that MI will enhance weight loss effectiveness as compared to conventional counseling*.

Table 1. Model Fidelity of Current MI program. Trained counselor's MI sessions were audiotaped and coded by 2 independent MI raters to examine proficiency with MI techniques. Recommended competency thresholds based on Motivational Interviewing Treatment Integrity Code (MITI) was used to determine model fidelity. Clinicians and raters also received weekly supervision with Dr. Bean. Intraclass correlations between the 2 raters ranged from 0.6-1.0

	MITI Ratings (SD)	Competency Threshold
Global Spirit	4.4 (0.42)	4.0
% Complex Reflection	74.4% (0.12)	50%
% Open Question	85.5% (0.09)	70%
% MI adherent	100% (0.00)	100%

3.1.2.2 Feasibility of Dietary Intervention. The PI's current NIH-funded study evaluates a putative insulin mediator during weight loss in obese women with and without PCOS. Dietary intervention *without* MI was used, as will be the case for the control group proposed. Of 40 women enrolled, 28 were African American, representing our local diverse population. Thirteen women dropped out, of whom 11 did so *before* their first visit, suggesting that readiness for change may have been a factor. Fourteen of 21 women with PCOS and 13/19 without PCOS completed the study. Mean weight loss was 3.5±3.3 kg. *These data confirm the dietary approach that we have developed will lead to weight loss. They also reiterate the need to address participants' readiness for change in attempting weight loss, which is expected to be bolstered by MI in this study.*

3.1.3. Study design. We will randomize 64 overweight / obese (BMI 27-40 kg/m^2) women with PCOS who desire pregnancy to MI counseling (n=32) or conventional counseling (control; n=32). Block randomization in blocks of 4 stratified by BMI (27-29.9, 30-34.9, 35-40 kg/m^2) will be used to ensure an even weight distribution between the 2 arms. After informed consent, both the MI and control groups will begin a 6-month lifestyle program as described in **Figure 1**, with an additional follow-up at 12 months to capture data on long-term

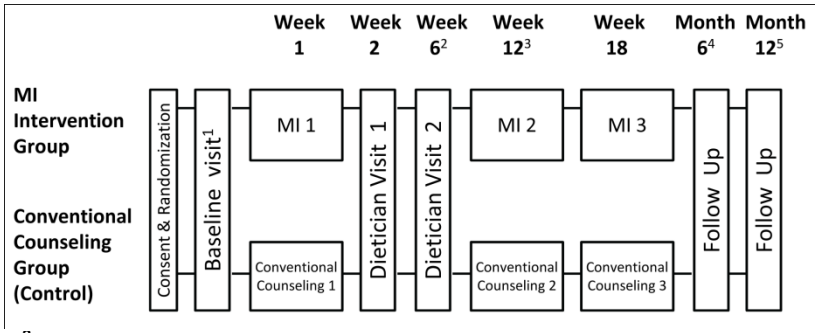


Figure 1. Study Design

MI 1, 2 &3 = Motivational Interview sessions 1, 2 and 3

Conventional Counseling 1, 2 & 3 = Attention control for contact frequency

Assessments & Procedures

[1]**Baseline Visit:** History and Physical; Menstrual history; Weight, height, waist & hip circumference; Diagnostic Labs; OGTT; 3-day food diary; Surveys for Aims 1 & 2.

[2]**Week 6:** Weight, waist & hip circumference.

[3]**Week 12:** Weight, waist & hip circumference; 3-day food diary; Repeat surveys for Aim 1.

[4]**Month 6:** Follow-up visit with physician; Weight, waist & hip circumference; 3-day food diary; Menstrual history and luteal-phase progesterone for cycling patients; Repeat surveys for Aim 1; Treatment satisfaction survey; Referral back to reproductive/ gynecological care per clinical indicators. [5]**Month 12:** Weight, waist & hip circumference; 3-day food diary; Menstrual history and luteal phase progesterone for cycling patients; Pregnancy status; Repeat surveys for Aim 1.

weight change and reproductive status. The study design was developed with translation to clinical practice in mind. In total, the MI Intervention group will receive 3 MI sessions by a psychology counselor and 2 dietician visits. The control group will receive the same 2 dietician visits. However, in lieu of MI counseling sessions, they will receive conventional counseling as an attention control.

3.1.4. Entrance criteria. Inclusion criteria: 1) Overweight/Obese (≥ 27 to 40 kg/m^2) premenopausal women between 21-35 years with PCOS. PCOS will be defined using criteria developed by the Androgen Excess and PCOS Society (37). 2) Willingness to attempt weight loss for 6 months before other fertility treatments. 3) No change in pharmacologic treatment of PCOS within 3 months. Exclusion criteria: 1) Diabetes mellitus by A_1c, fasting glucose or oral glucose tolerance test (OGTT); 2) Clinically significant pulmonary, cardiac, renal, hepatic, neurologic, infectious, neoplastic and malignant disease; 3) Participation in another weight loss program within 3 months; 4) History of weight loss surgery; 5) Pregnancy as documented by urine hCG.

3.1.5. MI Intervention.

Training of MI counselors: Two advanced psychology doctoral students (MI counselors) will be trained to deliver MI in one-to-one individual consultations. Training of the MI counselors will consist of a formal 2-day course in motivational interviewing, provided by a certified trainer from Motivational Interviewing Network of Trainers (38), followed by practice sessions (before the study), and weekly supervision and training (during the study) provided by Dr. Bean. We will use previously established methods to train and maintain MI competence and intervention fidelity in these MI counselors (see **3.1.2.1**).

MI sessions: Participants in the MI intervention arm will receive their first MI session within 1 week of their baseline visit. Each MI individual session will be 45-60 minutes in duration. During session 1, the following topics will be addressed: 1) participants' values on adoption of health behavior; 2) personal views on pros and cons of change, 3) barriers and facilitators to making these changes, and 4) goal-setting, if appropriate based on readiness to change. Open-ended questions and reflective listening will be used. MI will be integrated with the transtheoretical stages of change model so that the MI counseling will be appropriate for the individual's stage of change (39). During booster MI sessions 2 & 3, MI counselors will assist participants to set or advance individually tailored behavior goals. These sessions are also designed to engender motivation to sustain or initiate behavior change at different time periods when obstacles or motivations may have changed from those at the initial encounter (15;40). Participants will be assisted with relapse management and prevention.

Intervention fidelity: MI sessions will be audio-recorded and random 20-minute segments subjected to analyses. Two psychology students (not the study therapists) previously trained with the Motivational Interviewing Treatment Integrity (MITI) Code (41;42) will serve as raters. MI counselor's competence will be evaluated using MI proficiency guidelines (41). Ten percent of recordings will be double-rated to ensure consistent intraclass correlations between the 2 raters. MI raters will receive weekly supervision by Dr. Bean.

3.1.6. Dietician Visits. Dietary education will be provided by a registered dietician similar to the dietary intervention program currently utilized in Dr. Cheang's study (see **3.1.2.2**). Prior to consultation, participants will complete a 3-day food diary and a survey on weight and dieting history similar to one already in use. During Dietician Visit 1, specific dietary recommendations will be given based on information gathered from the baseline 3-day food diary and diet survey. The dietician will provide education on reducing overall caloric intake, portion size, overall and saturated fat intake, replacing simple with complex carbohydrates, and increasing fiber. In Dietician Visit 2, further adjustments and recommendations will be provided.

3.1.7. Conventional Lifestyle Counseling (Attention Control). To control for contact frequency, the control group will receive 3 sessions of conventional lifestyle counseling in lieu of the 3 MI sessions. The same MI counselors will provide these sessions. They will review standardized health information about PCOS with participants in a didactic format without using MI techniques. This style mirrors traditional usual care, providing information within an expert/recipient paradigm. Education about the recommendation to maintain healthy weight as part of PCOS treatment will be included, although specific motivational strategies for weight loss will not be provided during these sessions. All conventional lifestyle counseling sessions will be audio-recorded and coded with MITI 3.0 to ensure that MI techniques are not used during these sessions.

3.1.8. Outcome measures. (1) Weight loss: Weight, height, waist and hip circumference will be assessed at baseline, 6 and 12 weeks, and 6 and 12 months. Attrition rate and time to drop-out will also be assessed. (2) Adoption of health behavior: At baseline, 12 weeks, 6 and 12 months, participants will complete a 3-day food diary, and report their physical activity with validated Behavioral Risk Factor Surveillance System (BRFSS) physical activity questions (43). Estimates for total calories and macronutrient intake will be derived using the

Nutrition Data System for Research (University of Minnesota). Although not a main outcome, average minutes of moderate and vigorous physical activity per week will be obtained from the BRFSS responses. (3) Menstrual cyclicity: A menstrual history will be obtained at baseline and participants will be instructed to record the first day of menstrual bleeding every cycle during the study in a menstrual diary provided. At 6 and 12 months, a luteal-phase serum progesterone level will be obtained in all cycling patients to confirm ovulation. Although not a primary endpoint, pregnancy status will be ascertained at 12 months. (4) Psychological well-being: The following surveys will be administered at baseline, 6 and 12 months: i) SF-36, a widely used generic quality of life (QOL) instrument and its two composite physical and mental summary scores (44;45). ii) Quality-of-Life Questionnaire for Women with Polycystic Ovary Syndrome (PCOSQ), a PCOS- specific validated health-related QOL questionnaire assessing 5 domains, i.e. emotion, body hair, weight, infertility and menstrual problems (46;47). iii) Rosenberg Self-esteem Scale (48). The estimated time required for completing all surveys for Aim 1 are 25 minutes at baseline, 6 and 12 months, and 10 minutes at week 12.

3.1.9. Recruitment and subject accrual. Co-PI Dr. McGee is the Director of Reproductive Endocrinology and Infertility at Virginia Commonwealth University Health System (VCUHS), has an active practice, and supervises additional resident-based clinics. Also, VCUHS Gynecology and Endocrinology clinics, as well as the established "PCOS Research and Treatment Program" (www.vcu.edu/pcos) attract women with PCOS from across the Mid-Atlantic region. These clinics will provide an abundant patient population for recruitment into research studies. Dr. Cheang's current weight loss protocol (**3.1.2.2**) will have minimal overlap with this proposed study.

3.1.10. Sample size. The primary outcome is weight loss at 6 months. We consider a weight loss of at least 5% (12) to be clinically meaningful in the MI intervention arm. Assuming overweight / obese women with PCOS in North America weigh on average 100 kg (36), weight loss of 5kg would make results clinically meaningful. Current data in adults suggest a weight loss of 4.5-5.8 kg is realistic using the MI approach (20). For the control group, our preliminary data suggest mean weight loss of 3.5±3.3 kg without MI (but with weekly dietary follow-up). Since the proposed follow-up schedule is less intensive, we expect the control group mean weight loss to be 2.0±3.0 kg, as suggested by others (20). Hence, assuming that 6-month weight loss will be 5±3.5 kg in the MI group and 2±3.5 kg in the control group, we would need to study 23 women in each group to achieve a power of 0.8 with α=0.05. Attrition rates in PCOS weight loss studies have been about 40% traditionally (13;36), as compared to 4-18% in studies using MI (20;22). To be conservative, we assumed the higher 40% attrition rate in both groups. Therefore, we will recruit 32 women in each arm.

3.1.11. Data Analysis. MI intervention will be compared to the conventional counseling arm. The outcomes of interest will be assessed at 6 months, although 12 months of study period is planned so that we can gather preliminary data on pregnancy rates for future investigations. The main outcomes of interest are (1) change in weight from baseline to 6 months (primary outcome); (2) change in caloric and macronutrient intake, from baseline to 6 months; (3) resumption of ovulatory cycles at 6 and 12 months; (4) change in psychological well-being from baseline to 6 months as measured by SF-36, PCOSQ, and Rosenberg Self-esteem scale. Continuous variables not normally distributed will be log-transformed. To evaluate changes in continuous outcomes between the two groups, mixed model repeated measures ANOVA (with the treatment arm as an interaction term) will be used. This method uses all available data, even though some participants may drop out before the last observation time. For categorical variables, Chi-square tests will be used to compare between groups. $P<0.05$ will be considered significant.

3.1.12. Expected Outcomes. Weight Loss: We anticipate that MI counseling will promote increased weight loss at 6 months as compared to conventional counseling, in addition to lower attrition. The 1-year follow-up will provide additional data on the long-term maintenance of weight loss with these 2 approaches. We expect that women with PCOS who achieve weight loss, regardless of treatment assignment, will derive the following benefits: (i) Adoption of Health Behaviors: We anticipate women who lose weight will exhibit decreased caloric and fat intake. (ii) Menstrual Cyclicity: We expect weight reduction will lead to an increase in ovulatory cycles. (iii) Psychological Well-being: We anticipate that PCOS women who lose weight will have greater improvement in their QOL and self-esteem. We expect these indicators will be more evident in the MI group, as more participants in this group are expected to experience weight loss.

Results of the proposed research will provide evidence for a more effective approach to weight loss in overweight/ obese women with PCOS. With this advance, weight loss intervention can be more readily used as an initial or adjunctive management of PCOS-related infertility. Once we establish MI's efficacy, we can then evaluate how MI can be directly integrated into the clinical care of women with PCOS (e.g. through brief MI sessions or by training clinic providers in MI). Results will also form the basis of future studies on the effect of

adoption of health behaviors on psychological well-being, and ultimately, pregnancy rates. Obesity increases adverse outcomes in both the mother and fetus, so our findings may also inform preconception care.

3.1.13. Potential Problems & Alternative Strategies. Our working hypothesis for Aim 1 is that MI is superior to conventional counseling in effecting weight loss in obese women with PCOS, and successful weight reduction will lead to improved cyclicity and psychological wellbeing. Although our preliminary data (see **3.1.2.1**), and data from others on MI and weight loss (20;22;24;49), support the hypothesis for this aim, there is a possibility that MI may not offer additional benefits in overweight/ obese women with PCOS. In that unlikely event, we would turn to the extensive psychosocial and insulin dynamics assessments (Aim 2) to explain why PCOS women may be different from other adult populations in weight loss success with MI. In addition, should we observe that MI does not facilitate improved weight loss in PCOS women, we would have provided the first published documentation of its lack of efficacy in this population. Another potential drawback is that physical activity, although encouraged, is not a main focus of this study. Weight loss involves several behavior changes, which may not respond equally to MI. This proposal places a heavier emphasis on one of these behaviors, namely, adopting a healthy diet. Future proposals will study the role of MI in specifically encouraging physical activity. Finally, attrition is a problem for all weight loss studies. Subjects will be compensated appropriately for their time involvement. However, additional incentives, though effective in increasing compliance (50), will not be provided, as they could become additional motivating and confounding factors.

3.2. Specific Aim 2: Identify putative mediators and moderators of weight loss treatment effect

3.2.1. Rationale. Psychological morbidities are common in obese women with PCOS (51-55). Beginning evidence suggests that psychological distress may affect adherence to weight loss interventions (56). However, factors underlying weight loss treatment effect in obese women with PCOS are largely unknown. Better understanding of the factors associated with weight loss will lead to the development of more targeted intervention strategies, to directly address mechanisms of change and tailor treatment approaches. For example, if findings suggest motivation is a moderator of treatment response, and that simple advice-giving is effective in already motivated women with PCOS, this simpler approach may be more cost-effective than MI. In this aim, *hypothesis-generating analyses* will be conducted to examine putative moderators and mediators of treatment effect. Based on our preliminary data (see **3.2.2**), we expect that moderators and mediators of weight loss treatment effect in obese women with PCOS exist. Major mediators and moderators identified will inform the design of subsequent large-scale trials to evaluate whether it is possible to use these characteristics to tailor treatment approaches, and whether treatment can be fine-tuned to increase effectiveness.

3.2.2. Preliminary Studies

Table 2: Predictors of weight loss in obese women with and without PCOS in PI's current weight loss study

Positive Predictors of Weight Loss Success	OR	P-value
Protein intake at baseline (g/day)	1.07	0.0496
Prior weight loss group programs (Y/N)	4.80	0.0357

Negative Predictors of Weight Loss Success	OR	P-value
Prior use of weight loss medications (Y/N)	0.21	0.0496
Low cognitive restraint	0.59	0.0334
AUC insulin (μIU/ml • 120 min)	0.83	0.0197

Methods: Baseline characteristics and insulin dynamics measures were obtained before an 8-week dietary weight loss program. Weight loss success was defined as a minimum loss of 3.6kg (8 lbs) over 8 weeks. Odds ratio estimates of weight loss success were presented per unit of independent variable shown. Low cognitive restraint was defined as the lowest tertile of the Three Factor Eating Questionnaire cognitive restraint subscale.

Although data in Table 2 were derived from both obese PCOS and non-PCOS women, *these data validate Aim 2's underlying concept that moderators and mediators of weight loss treatment effect exist*. The proposed studies in Aim 2 will determine the mediators and moderators of weight loss specifically in obese women with PCOS desiring pregnancy.

3.2.3. Study Design, Entrance Criteria, Interventions, Attention Control. Aims 1 and 2 are conducted concurrently as a single study. Thus, study design and interventions are identical to Aim 1.

3.2.4. Biochemical and insulin dynamics measurements. At baseline before weight loss interventions, participants will arrive at the General Clinical Research Center at 0800h after a 12-hr overnight fast. Fasting blood samples will be drawn at 0815h and 0830h, and pooled for determination of plasma insulin, glucose, sex steroids (testosterone, androstenedione, DHEA-sulfate, estradiol, estrone, progesterone) and SHBG. At 0900h, an oral glucose tolerance test (OGTT) will be performed with 75 g oral dextrose and blood samples will be collected for serum glucose and insulin every 15 min for 2h. The respective responses will be analyzed by calculating the areas under the response curves (AUC) by the trapezoidal rule. Peripheral insulin sensitivity will be assessed by the Matsuda index (57). Early insulin secretion will be estimated by dividing the increment in

serum insulin by the increment in plasma glucose from 0-30 min of the OGTT (I_{0-30}/G_{0-30}) (58). β-cell function will be assessed by AUC insulin. β cell compensation for insulin resistance will be calculated by the disposition index (product of [I_{0-120}/G_{0-120}] and the Matsuda index).

3.2.5. Mediators/ Moderators. Putative moderators and mediators to be examined include: 1) Demographics (e.g. race, education level, socio-economic status); 2) Hyperandrogenemia, and insulin sensitivity / secretion parameters (**3.2.4**); 3) Number and methods of previous weight loss attempts from the diet history survey (**3.1.6**), baseline macronutrient intake and physical activity (**3.1.8**); 4) Treatment assignment; 5) Treatment liking, as assessed by a program satisfaction survey at 6 months (59); 6) Counselor-patient relationship, as assessed by the Working Alliance Inventory (WAI) by both the counselor and participant (counselor version and client version) (60) after each MI and conventional counseling session; 7) Depression at baseline as assessed by the Beck Depression Inventory 13-item short form (61); 8) Eating behavior (hunger, disinhibition, cognitive restraint), as assessed by the Three Factor Eating Questionnaire (62;63) at baseline; 9) Social support at baseline as assessed by the Medical Outcomes Study Social Support survey (64); 10) Body dissatisfaction, as assessed by the Figure Rating Scale (65) and the Body Features Satisfaction scale modified for PCOS (66); 11) Readiness to change at baseline as assessed by Weight Loss Behavior-Stage of Change Scale (67); 12) Pregnancy desire, intention and planning as assessed by a validated instrument (68). Estimated time needed to complete the above surveys is approximately 25 minutes (for items 7-12), 5 minutes (for the WAI after each counseling session), and 5 minutes (for the program satisfaction at 6 months).

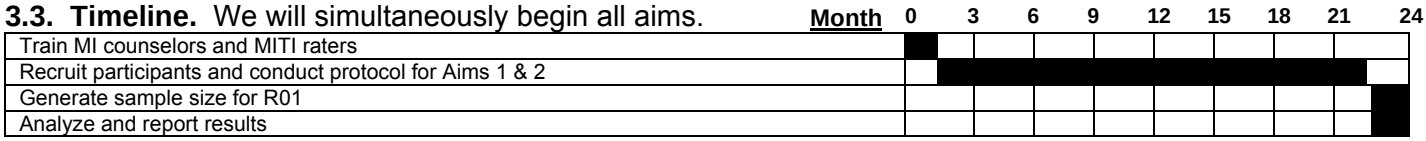
3.2.6. Recruitment, subject accrual and sample size. There are no current data reporting moderators or mediators of weight loss in women with PCOS, so sample size specific to Aim 2 cannot be estimated, and will be the same as Aim 1. Data obtained from Aim 2 will be used to estimate sample size for future investigations.

3.2.7. Data Analysis. It is challenging to evaluate mediators and moderators of treatment using this small sample in a two-group study. We will use Kraemer et al.'s recommended methods (69) to identify major potential confounders as mediators, moderators, and risk factors. This approach will generate hypotheses to be examined in a larger randomized trial, if warranted. Weight loss will be evaluated as a continuous variable. The independent variables as presented in **3.2.5** will be examined. Due to the small sample size these will each be examined individually without correction for multiple comparisons. Although use of this approach could generate false positives, these are simply exploratory hypotheses to be examined more rigorously in subsequent projects.

3.2.8. Expected Outcomes. We anticipate that novel mediators and moderators of weight loss treatment effect in overweight/obese women with PCOS will be identified. These results will be used in *prospective* studies to design and evaluate more targeted intervention strategies to directly address mechanisms of change, and tailor treatment approaches.

3.2.9. Potential Problems & Alternative Strategies. It is possible that mediators or moderators identified, even if significant, may not be clinically meaningful, because weight loss is evaluated as a continuous variable in this aim. We are aware that weight loss of at least 5% is a minimum threshold for improvement in reproductive function (12) and important health benefits (70). Major moderators and mediators will be confirmed using weight loss of 5% as a categorical dependent variable. Analyses in this aim are exploratory in nature and will only be used to inform future study designs.

3.3. Timeline. We will simultaneously begin all aims.



3.4. Future Directions. Our *long term goal* is to elucidate effective therapies to ameliorate the fertility and metabolic consequences for women with PCOS. At the conclusion of the proposed studies, we would have developed data supporting or refuting the role of MI in facilitating weight loss in overweight/obese women with PCOS seeking to conceive. The putative mediators and moderators of weight loss identified will inform future study designs to develop more targeted intervention strategies, to directly address mechanisms of change and tailor treatment approaches. Also, we will study the effect of integrated MI delivered at patients' clinic encounters. Together, we expect these investigations will lead to weight loss approaches that are translatable into clinical practice, and thus address a pressing clinical need (12;71). Most importantly, results of this study will form the basis for future investigations on the effect of adoption of health behaviors and improvement in psychological well-being on fertility and pregnancy rates (26).