Association between β2-adrenoceptor (ADRB2) haplotypes and insulin resistance in

PCOS

Short Title: *ADRB2 and insulin resistance in PCOS*

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Abstract:

Objetive: The aim of this study was to explore β2-adrenoceptor (ADRB2) haplotype associations with phenotypes and quantitative traits related to insulin resistance (IR) and the metabolic syndrome (MS) in a polycystic ovary syndrome (PCOS) population. A secondary purpose was to assess the association between ADRB2 haplotype and PCOS.

Design: Genetic polymorphism analysis. Cross sectional case-control association study.

Setting: Medical University Hospital and research laboratory.

Patients: 165 unrelated women with PCOS and 116 unrelated women without PCOS (control sample).

Measurements: Clinical and biochemical measurements, and ADRB2 genotyping in PCOS patients and control subjects.

Methods: ADRB2 haplotypes (comprising rs1042711, rs1801704, rs1042713 and rs1042714 in that order), genotyping and statistical analysis to evaluate associations with continuous variables and traits related to IR and MS in a PCOS population. Associations between ADRB2 haplotypes and PCOS were also assessed.

Results: We observed an age-adjusted association between ADRB2 haplotype CCGG and lower insulin (p=0.018) and HOMA (p=0.008) in the PCOS sample. Interestingly, the

expected differences in surrogate measures of IR between cases and controls were not significant in CCGG/CCGG carriers. In the case-control study, genotype CCGG/CCGG was associated with a 14% decrease in PCOS risk (p=0.043), taking into account confounding variables.

Conclusions: Haplotype I (CCGG) has a protective role for IR and MS in PCOS.

Introduction:

Polycystic ovary syndrome (PCOS) is recognised as the most common endocrine disorder in women of reproductive age (1,2,3). Insulin resistance in these patients may play an important role in the development of the syndrome where insulin has an influence on LH and steroidogenesis. Besides, many PCOS women are obese and develop a number of complications associated with obesity. Genetic variants which influence insulin response and/or obesity may be related to increased prevalence of PCOS.

The pathophysiological mechanisms responsible for the development of obesity and insulin resistance (IR) in PCOS are multifactorial and abnormal regulation of adipocyte lipolysis might be a key component. Obesity is associated with an increase in basal lipolysis (4) but a decrease in catecholamine-stimulated lipolysis (5,6) where the beta-adrenergic receptor plays a major role (4). In PCOS, catecholamine-induced lipolysis is reduced in subcutaneous (sc) fat (7) but increased in visceral fat (8), resulting in an increase in lipid content in fat cells and excess of fatty acid delivery to the liver which could lead to hyperinsulinaemia, glucose intolerance, and dyslipidaemia (9,10). Such lipolysis defects are identical to those observed in the metabolic syndrome (MS) so it could be a primary pathogenic mechanism for the development of the three disorders: obesity, PCOS and MS.

Insulin resistance and concomitant hyperinsulinaemia are common features in PCOS patients (50-70%)(11), and, together with obesity (12), are frequent pathogenetic factors in MS. Moreover, MS frequency is increased in PCOS women compared with age- and weight-matched controls (13,14) with an overall prevalence of 43-47% (15), especially in women with high insulin levels and BMI (16).

An obesogenic lifestyle seems to be the main factor underpinning common variants of obesity and insulin resistance; however, the role of genetic predisposition in various conditions, including PCOS, is increasingly recognized (17,18).

We studied the β2-adrenoceptor (ADRB2) owing to its dominant effect as lipolytic receptor in human white adipose tissue (19) and skeletal muscle (20). Recently Masuo et al (21) reviewed the close relationships between β2- and β3-adrenoceptor polymorphisms accompanying elevated sympathetic nervous activity, blood pressure elevation, weight gain, and insulin resistance.

The aim of this study was to explore associations between ADRB2 haplotypes and phenotypes and quantitative metabolic traits related to obesity, lipid metabolism and IR in a PCOS population. A secondary purpose of the present study was to perform a cross sectional case-control association study between PCOS and ADRB2 haplotypes.

Subjects and methods:

- Subjects

220 women with PCOS were recruited from outpatients attending the Endocrine Division of Hospital Durand, Buenos Aires (2006-2010). A sample of 165 unrelated women was selected for the molecular study according to the availability of metabolic and anthropometric parameters, informed consent and the absence of consanguinity.

PCOS was defined by 1) the presence of hyperandrogenaemia or clinical hyperandrogenism, 2) oligo-ovulation, and 3) the exclusion of other disorders, as described by the NIH 1990 criteria (22).

Patients who had any of the following illnesses, conditions or requirement were excluded: Cushing's syndrome, 21-hydroxylase deficiency, thyroid dysfunction, hepatic or haematological disease, diabetes, or hyperprolactinaemia.

Controls were unrelated healthy females, without clinical components of PCOS or family history of PCOS (self reported), with normal weight, waist circumference <88cm, normal findings on medical examination and blood count, and not taking any medication. Volunteers were Argentinian of self-reported European ancestry, particularly from southern European countries (Spain and Italy), living in Buenos Aires metropolitan area, randomly recruited between April 2007 and April 2009.

This work was carried out in accordance with the Declaration of Helsinki, and approved by the Hospital ethics committee. All subjects gave their written consent.

-Clinical measurements

Anthropometric measurements (height, weight and waist circumference) were determined by a standardized protocol in every subject. Waist circumference (WC) was measured to the nearest 0.1cm, with an inelastic fibreglass standard tape, in a horizontal plane, over the unclothed abdomen, at the narrowest point between the costal margin and iliac crest. Measurement was made at the end of a normal expiration. Systolic (SBP) and diastolic (DBP) blood pressures were recorded using a standard mercury sphygmomanometer after at least 10 min of rest.

Blood samples were drawn after a 12h overnight fast. Total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were determined in serum by enzymatic methods using commercial kits (TG Triglycerides GPO-PAP, CHOL Cholesterol CHOD-PAP and Phosphotungstate Precipitant, Roche Diagnostics, Mannheim, Germany) in a Hitachi 727 autoanalyzer. Intra-assay coefficients of variation (CVs) for TG and TC were 1.3 and 1.1%, respectively. Inter-assay CVs were 2.4% and 1.5%, respectively. Fasting plasma glucose (FPG) was determined by a glucose-oxidase method (GLU Glucose GOD-PAP, Roche Diagnostics, Mannheim, Germany) in a Hitachi 727 autoanalyzer. Intra- and inter-assay CVs were 0.9 and 1.8%, respectively. Fasting serum insulin (FI) was measured by radioimmunoassay (RIA) with a commercial kit (Human Insulin Specific RIA kit, Linco Research Inc., St. Louis, MO, USA) by using a gamma counter (DPC Gambyt CR, Los Angeles, USA), with a lower detection limit of 2 mU/L, intra- and inter-assay CVs being <1 and <7.43%, respectively and cross-reactivity less than 0.2% for intact human proinsulin. Homeostasis Model Assessment (HOMA) was calculated with the following formula: basal glucose mmol/L x basal insulin mIU/L/22.5.

-Phenotype assessment

In Table 1 we showed significant differences in continuous variables between PCOS and control samples (BMI, WC, SBP, DBP, FG, TC, TG, HDL-C, TG/C-HDL, fasting insulin). Each subject was assessed for the presence of 1) MS using the AHA/NHLBI (American Heart Association/National Heart, Lung and Blood Institute) criteria **(23)** [any 3 or more of the following: WC $\geq$ 88 cm, fasting TG $\geq$ 1.7mmol/L, SBP $\geq$ 130 mmHg, and/or DBP $\geq$ 85 mmHg, fasting HDL-C $\leq$ 1.03mmol/L, and FPG $\geq$ 5.6mmol/L]; 2) obesity (BMI $\geq$ 30.0

kg/m^2) **(24)**; 3) abdominal obesity (waist circumference ≥ 88 cm), 4) hypertriglyceridaemia (HTG) (fasting TG ≥ 1.7mmol/L), 5) hypertriglyceridaemic waist (HW) (TG ≥ 1.7mmol/L + WC ≥ 88cm); 6) decreased HDL-C (fasting HDL-C <1.03mmol/L); 7) impaired fasting glucose (IFG= Fasting plasma glucose ≥ 5.6mmol/L), 8) IR (insulin resistance) estimated by 75th percentile HOMA cutoff point (HOMA>2.3=IR).

-*Genotyping*

As described previously **(25)**, we developed a simple method of genotyping ADBR2 haplotypes composed of rs1042711, rs1801704, rs1042713 and rs1042714. The rs1042711 (-47 C/T) and rs1801704 (-20 C/T) are located in the 5' leader cistron and rs1042713 (c.46A>G, Arg16Gly, R16E) and rs1042714 (c.79C>G, Gln27Glu, Q27E) in exon 1. It was possible to identify three major haplotypes (I, II and III) considering the different combinations of the four polymorphisms presented in the amplified fragment.

Data from International Haplotype Map Project (http://hapmap.ncbi.nlm.nih.gov/) for European Americans from Utah (HapMap-CEU) and the software Haploview 4.1 **(26)** provided us tag-SNPs in LD; with a minor allele frequency (MAF) ≥0.10, minimum r^2 ≥0.8, and minimum LOD =3.0.

Briefly, genomic DNA was isolated from peripheral blood according to standard procedures. PCR was carried out to amplify a 353 bp region of ADBR2, single-strand conformation polymorphisms (SSCP) gel electrophoresis was performed and visualized with silver staining. The PCR-SSCP procedure was validated with automated sequencing. Genotyping accuracy was assessed by inclusion of duplicates and controls.

-Statistical analysis

Analyses were done separately for haplotypes and followed up by analysis of individual SNPs. Quantitative data were expressed as means ± SE. For comparison of continuous variables we conducted one-way ANOVA with Levine's Test for equality of variances. Otherwise, we used Welch test. Regression analyses were used to adjust for possible confounding variables (age, BMI).

The effects of a particular haplotype load (0= no copies, 1= 1 copy; and 2= 2 copies of the particular haplotype) on continuous or categorical variables were tested using linear or logistic regression respectively.

Deviation of the genotype distribution from the Hardy–Weinberg Equilibrium (HWE) was tested using Chi square test for each SNP, for both samples.

For individual SNP association analyses, we performed 1-df (1 degrees of freedom) test of association (dominant and recessive genetic models were tested).

Differences between prevalence rates were assessed by Fisher exact test.

We calculated odds ratio (OR) and 95% confidence intervals (95%CI) when appropriate.

A p-value less than 0.05 was considered statistically significant. Statistical analyses were conducted using the program for Statistical Package for the Social Sciences, version 13.0 for Windows (SPSS, Inc., Chicago, IL).

Results

The prevalence of MS and related traits in PCOS were as follows: MS (using AHA/NHLBI criteria) 20.2%; Obesity 42.1%; abdominal obesity 52.8%; HTG 25.0%; HW 19.6%; decreased HDL-C 21.9%; IFG 11.5%. Simultaneously the prevalences of IR defined by HOMA for lean and obese PCO patients respectively (34% and 70%) were determined. In

Table 1 we showed the significant differences in continuous variables observed between PCOS and control samples (BMI, WC, SBP, DBP, FG, TC, TG, HDL-C, TG/C-HDL, fasting insulin).

We identified the 3 main haplotypes that we previously observed in another Argentinian sample **(25)**, which correlated with the haplotypes first described by Drysdale et al **(27)**. The frequencies of the three haplotypes (rs1042711, rs1801704, rs1042713 and rs1042714 in that order) were: CCGG (I) 0.31; TTAC (II) 0.38 and TTGC (III) 0.31.

We identified the 6 genotypes CCGG/CCGG (I/I), CCGG/TTAC (I/II), CCGG/TTGC (I/III), TTAC/TTAC (II/II), TTAC/TTGC (II/III) and TTGC/TTGC (III/III).

- ADRB2 haplotype frequency distributions according to phenotypes related to MS in PCOS

We compared the overall frequency differences (3x2 contingency table) across the three possible haplotypes and the different phenotypes, and observed a borderline association between haplotype I (CCGG) and MS (p = 0.051; OR = 0.41, 95 % CI = 0.17 - 1.01). We could not find any significant association with obesity, abdominal obesity, IFG, HTG, decreased HDL-C, HW or IR phenotypes.

To further explore the effect of the haplotype I load (0= no copies, 1= 1 copy; and 2= 2 copies of the particular haplotype) we performed logistic regression analysis. We found a statistically significant association between haplotype I and MS (p = 0.042; OR= 0.37 (95 % CI = 0.14 - 0.97) but differences were no longer significant after taking into account age (p = 0.077). No other significant associations were found between haplotype load and phenotypes under study in the PCOS sample.

Haplotype I was less represented in the PCOS IR-subgroup (HOMA>2.3) (haplotype I frequencies in IR and non IR women were 0.27 and 0.37, respectively) and we observed an association with insulin resistance taking into account age (p=0.083 and adjusted-p=0.033). Although haplotype I was less represented in the PCOS obese-subgroup (haplotype I frequency in obese and non obese individuals were 0.18 and 0.35, respectively) we did not observed any association with obesity (p=0.086 and p= 0.229 when age adjusted).

- Haplotype load - association analysis of quantitative metabolic traits in PCOS

The effects of haplotype I load on quantitative metabolic traits are represented in Figure 2. In PCOS, the correlation between decreased values of BMI, insulin, HOMA, TG/HDL-C, WC and BMI, and increase in haplotype I (X/X, I/X and I/I) load are shown.

We performed linear regression analysis to assess the effects of a particular haplotype load on quantitative metabolic traits. We found an association of haplotype I load (CCGG) with BMI (p = 0.040), insulin (p = 0.015) and HOMA (p = 0.017). Differences in insulin and HOMA remained significant taking into account age (p=0.018 and p=0.008, respectively) and after adjusting for age and BMI (p=0.032 and p=0.014, respectively).

We did not find any other significant regression association between haplotype I and lipid and/or other obesity-related variables in PCOS patients (waist circumference, triglycerides and TG/HDL-c). Data not shown.

No significant association of haplotypes II (TTAC) or III (TTGC) with continuous variables was apparent.

Case – control association study between ADRB2 haplotypes and PCOS

We did not find any evidence of association when comparing overall frequency differences across the three possible haplotypes between cases (PCOS) and controls (p=0.084, Figure 1).

We performed logistic regression analysis to assess the association between haplotype I load and presence of PCOS. We included in the model the variables previously associated with haplotype I in the PCOS sample (fasting insulin, HOMA-IR and MS) and age. Association of haplotype I with PCOS was significant under a recessive model (I/I vs. I/X + X/X) (p = 0.043; OR = 0.14, 95 % CI = 0.02 - 0.93).

The same study was performed with non insulin resistant PCOS (HOMA<2.3 by 75th percentile HOMA value) compared to the control group (non insulin resistant) and we observed a significant association of haplotype I with PCOS under a recessive model (I/I vs. I/X + X/X) (p = 0.004; OR = 0.026, 95 % CI = 0.002 - 0.32).

Taking into consideration the intra PCOS association study performed previously, we analyzed the contribution of haplotype I load to quantitative traits related to obesity, lipid metabolism and surrogate measures of IR, between cases (PCOS) and controls. Figure 2 shows that the PCOS genotype X/X carriers' subgroup (without haplotype I) had significantly higher surrogate measures of IR (insulin, HOMA, TG and TG/HDL-C ratio) than controls (p < 0.005 adjusted by age and BMI). The genotype I/X individuals showed the same results but, interestingly, in the I/I genotype carriers' subgroup, differences between cases and controls were not significant. The same results were observed when

analysis was performed between non IR PCOS and non obese PCOS vs. controls (non IR and non obese, respectively), data not shown.

WC and BMI were compared, and statistically significant differences were observed between cases and controls, independently of haplotype I presence (p < 0.005 adjusted by age and BMI, Figure 2).

- *Single Locus Analysis*

We only performed single locus analysis of rs1042713 and rs1042714. rs1042711 in our sample seemed to be linked to rs1801704 and rs1042714 since we only found three haplotypes (CCGG, TTAC and TTGC, I, II and III respectively). Also, considering the linkage disequilibrium (LD) analysis with HapMap-CEU data, we observed that rs1042714 was in linkage disequilibrium (LD) with rs1801704 ($r^2 = 0.96$) (Figure 3).

Allelic frequencies were as follow: in the control sample: rs1042713 G 0.59 and A 0.41; rs1042714 C 0.68 and G 0.32; and in the PCOS sample: rs1042713 G 0.62 and A 0.38; rs1042714 C 0.69 and G 0.31. Both SNPs were in concordance with HWE as follows: rs1042713: Controls $\chi^2 = 0.04$, p = NS; PCOS $\chi^2 = 0.17$, p = NS; rs1042714: Controls $\chi^2 = 1.17$, p = NS; PCOS $\chi^2 = 0.31$, p = NS.

We did not find significant associations of rs1042713 with categorical or continuous variables in the PCOS sample. rs1042714 was not associated with phenotypes (categorical variables) under study in the PCOS sample but rs1042714 GG carriers showed lower fasting insulin (p = 0.022) and HOMA (p = 0.030) compared with allele C carriers (recessive model). Differences in insulin and HOMA remained significant after taking into

account age (p = 0.021 and p = 0.010, respectively) and after adjusting for age and BMI (p = 0.035 and p = 0.017, respectively) in the PCOS sample.

rs1042713 and rs1042714 allele and genotype frequencies did not differ significantly between PCOS cases and control subjects.

Discussion

This is the first report that explores ADRB2 haplotypes (rs1042711, rs1801704, rs1042713 and rs1042714) for their association with phenotypes and quantitative metabolic traits related to IR, obesity and lipid metabolism, in a Caucasian PCOS population. Beta adrenergic receptor activity was recently identified in the molecular ontology genes within the protein network constructed by Mohamed-Hussein and Harun, demonstrating a probable role of the gene in the pathophysiology of PCOS **(28)**.

We observed an association between ADRB2 haplotype CCGG (I) and absence of MS (significant) or insulin resistance (borderline) in PCOS women; results were confirmed by haplotype I load logistic regression. Although haplotype I was less represented in the PCOS obese-subgroup we did not observe any association with obesity. Furthermore, no associations with abdominal obesity, IFG, HTG, decreased HDL-C, HW or IR phenotypes were found.

In PCOS patients, decreased values of BMI, insulin, HOMA, TG/HDL-C, WC and BMI correlated with increased haplotype I load (X/X, I/X and I/I). Particularly, we observed linear regression association of haplotype I load (CCGG) with lower insulin and HOMA.

A case–control association study between ADRB2 haplotypes and PCOS was also performed, and we observed that CCGG/CCGG carriers had significantly lower risk of developing PCOS taking into account fasting insulin, HOMA-IR, MS and age. Genotype

CCGG/CCGG was associated with a 14% decrease in PCOS risk taking into account these confounding variables. This may therefore indicate that women who shared one or more of the confounding parameters analyzed, like fasting insulin, HOMA-IR, MS and the same age, would have a lower risk of developing PCOS if they carried two copies of haplotype CCGG. Interestingly, cases and controls within the CCGG/CCGG-carrier group had no significant differences in surrogate measures of IR (insulin, HOMA and TG/HDL-C ratio).

In our previous study in another Argentine population **(25)**, we found statistically significant frequency differences for haplotype CCGG/CCGG between obese and normal weight subjects (p: 0.0348, odds ratio: 0.2487, CI 95%: 0.07-0.91) indicating a possible protective role of the haplotype CCGG/CCGG for obesity. In the PCOS sample studied in this work, although haplotype CCGG was less represented in obese PCOS women, no significant differences attributable to the ADRB2 haplotypes were observed for WC and BMI.

Taking all the results together, haplotype CCGG appears to have a protective role for IR, and MS in PCOS, regardless of age. Although the present study provides exploratory results and should be confirmed in a second and independent replication cohort, previous association studies have shown conflicting results regarding ADRB2 haplotypes and different phenotypes, as recently analyzed in a meta-analysis **(29)**. Ethnic influences and differences in the degree of the phenotypes among research subjects would explain such discordances **(30)**.

The vast majority of association studies which have considered ADRB2 as a candidate gene have analyzed one or both of the non-synonymous coding polymorphisms (rs1042713, Arg16Gly and rs1042714, Gln27Glu) in different diseases. In an attempt to explore the association signal, we performed analysis with individual SNPs. rs1042714 GG

(Glu27/Glu27) genotype carriers showed lower fasting insulin and HOMA compared with allele rs1042714C carriers (Gln27/Glu27 or Gln27/ Gln27) even after adjusting for age and BMI in the PCOS sample. Kurabayashi et al (31) reported that women with rs1042713AA (not GG, Arg16/Arg16) genotype or with either rs1042714CG (Gln27/Glu27) or GG (Glu27/Glu27) genotype had significantly higher insulin resistance than those with the rs1042713GG (Gly16/ Gly16) and rs1042714CC (Gln27/ Gln27) genotypes, in a Japanese PCOS sample.

We did not find allelic association with PCOS in the sample studied. In the previous case-control study, conducted by Kurabayashi et al. (31) (fifty-nine women with PCOS and 97 controls), the rs1042714G allele frequency was shown to be significantly higher in PCOS patients when compared to controls, while the rs1042713G allele frequency was only slightly lower in the PCOS group as compared to controls. In contrast, we observed that the rs1042713 G allele frequency was only slightly higher in the PCOS group as compared to controls (0.62 vs. 0.59) and no differences were observed for the rs1042714 G allele (0.32 vs. 0.31). Contradictory results are probably due to the different allele frequencies observed (HapMap-JPT/CEU allele distribution, http://hapmap.ncbi.nlm.nih.gov/) or different LD structure in both populations. Furthermore, discrepancies in results have been reviewed by Masuo (6) in relation to ADRB2 polymorphisms and obesity, glucose intolerance, hypertension and/or diabetes. It must be considered that SNPs may be acting together in specific haplotype pairs (32) although each may have a different modest effect (32, 33, 34, 35) and might address these inconsistencies.

Of note, a gene-wide association study (GWAS) of the ADRB2 candidate region, by means of the haplotype tagging strategy, would require genotyping rs1042713, rs1042714, rs1042718 and rs1042719, to capture 100% of alleles with minor allele frequency ≥ 0.10

(Figure 3). However, tagging only common SNPs, as we have done, excluded the possibility of discovering any substantial effect associated with low frequency SNPs.

In conclusion, a protective role of haplotype I in insulin resistance would influence the development of PCOS, and other related metabolic disorders such as MS. Considering the complexity and heterogeneity of PCOS **(32),** and the possible pathogenetic role of the A*DRB2* gene, A*DRB2* haplotype genotyping in the early stages of PCOS could help to provide a risk predisposition assessment for IR and MS.

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Table 1. Clinical characteristics

Measure	Control women	PCOS women	P	P[a]
n	121	165		
Age	30.,78 ± 0.78	26.,42 ± 0.50	<0.001	NA
BMI (kg/m^2)	21.08 ± 0.18	29.28 ± 0.63	<0.001	<0.001
WC (cm)	71.05 ± 0.68	89.32 ± 1.61	<0.001	<0.001
SBP (mmHg)	109.73 ± 2.33	111.23 ± 1.78	NS	NS
DBP (mmHg)	72.75 ± 0.91	72.22 ± 1.38	NS	NS
FG (mmol/L)	4.5080.92 ± 0.074	5.0791.39 ± 2.630.15	<0.001	<0.001
T-C (mmol/L)	4.25164.31 ±	188.794.88 ±	<0.001	<0.001
TG (mmol/L)	67.000.76 ± 2.890.03	123.591.40 ±	<0.001	<0.001
HDL-C (mmol/L)	56.781.47 ± 1.430.04	51.321.33 ± 1.470.04	0.010	0.025
TG/HDL-C	1.22 ± 0.06	2.,80 ± 0.24	<0.001	<0.001
Fasting Insulin	5.65 ± 0.27	14.27 ± 0.86	<0.001	<0.001
HOMA	1.09 ± 0.05	3.23 ± 0.22	<0.001	<0.001

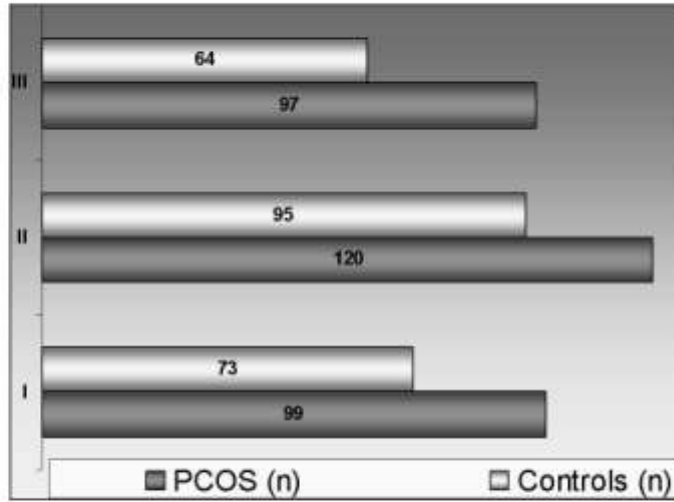Figure 1: ADBR2 haplotype distribution between cases and controls

Figure 2: Contribution of haplotype I load to quantitative traits related to obesity and lipid metabolism in PCOS and controls
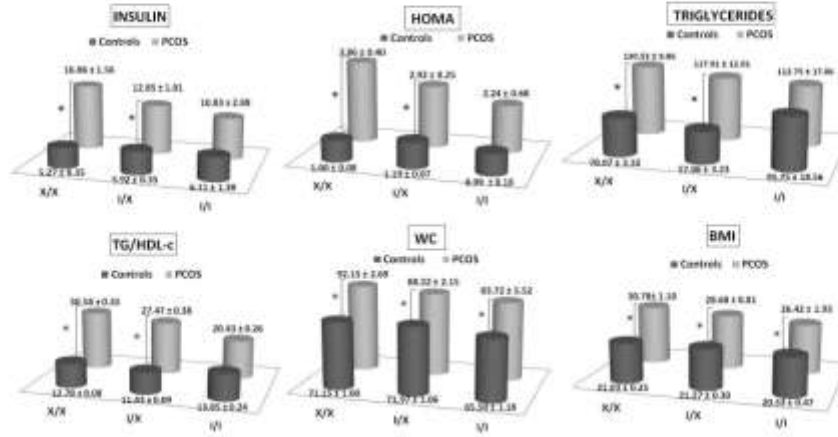
Figure 3: Schematic representation of LD throughout ADRB2 gene