Genetic variability in the cholecystokinin A receptor affects lipid profile and glucose tolerance in patients with polycystic ovary syndrome

Keywords
polymorphism, PCOS, cholecystokinin, CCKAR, CCKBR

Abstract
Introduction
Cholecystokinin (CCK) is involved in several metabolic pathways and CCK agonist are considered as potential novel treatment option in populations with increased metabolic risk, including polycystic ovary syndrome (PCOS). As genetic variability of cholecystokinin A and B receptor genes (CCKAR and CCKBR, respectively) may modify its biological actions, we investigated the impact of CCKAR and CCKBR genetic variability on anthropometric and metabolic parameters in patients with PCOS.

Material and methods
Our cross-sectional study included 168 patients with PCOS and 82 healthy female controls genotyped for polymorphisms in CCKAR (rs6448456 and rs1800857) and CCKBR (rs2929180, rs1800843, rs1042047 and rs1042048) genes.

Results
The investigated polymorphisms were not associated with anthropometric characteristics of patients with PCOS, however, among healthy controls carriers of at least one polymorphic CCKBR rs1800843 allele had bigger waist circumference (p=0.027) and more visceral fat (p=0.046). Among PCOS patients carriers of at least one polymorphic CCKAR rs6448456 C allele had significantly higher total blood cholesterol and LDL, and significantly lower blood glucose levels after 30, 60 and 90 minutes of the oral glucose tolerance test (all p<0.05). Healthy controls with at least one polymorphic CCKAR rs1800857 C allele were less likely to have high metabolic syndrome burden (p=0.029).

Conclusions
Genetic variability in CCKAR affects lipid profile and post-load glucose levels in patients with PCOS and is associated with metabolic sydrome burden in healthy young women. Further investigation of the role of genetic variability in CCKAR and CCKBR could contribute to development of individually tailored treatment strategies with CCK receptor agonists.

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Explanation letter
In the revised manuscript, all the questions and comments of the Reviewers were taken into consideration and the revised manuscript incorporates all due revisions and explanatory comments. As healty subjects were included in the study and additional experimental work had to be performed, two more authors were invited to participate in the study and in preparation of the revised manuscript.

Reviewer 1:
Q: Possible change. Line 175: decreased instead of deceased
A: Thank you for noticing this typing error, we have corrected it.

Reviewer 2:
Q: the importance of the conclusion observed on one tested group, in one-time point must be confirmed by comparing it to a group of non-PCOS patients.
A: We have carefully evaluated this comment regarding the lack of a non-PCOS group and we were

very fortunate that due to our previous collaborations we could include a group of young female controls for which we also had some basic anthropometric and metabolic data as compared to our usual control group of healthy female blood donors for which we have no other data except for age. As this control group was not recruited at the same time and under the same study protocol as PCOS patients, the clinical and laboratory data are not directly comparable between the two groups, therefore the results are presented in different tables. We really have to thank the reviewer for persisting in inclusion of a non-PCR group as, although this resulted in additional experimental work and delayed the resubmission of the manuscript, the inclusion of these healthy controls provided very interesting novel data and had improved the manuscript significantly.

Reviewer 3:
Q: The paper is very interesting and well written. I only suggest to discuss if this polymorphism may affect the response to insulin therapy as in psoriatic patients receiving TNF alpha inhibitors (see and add as references papers by Murdaca et al concerning pharmacogenomics and etanercept in psoriatic patients published in Journal of investigative dermatology and in expert opinion on drug metabolism)
A: We appreciate pointing out that receptor polymorphisms may also play a role in treatment response. We have included a following sentence, supported by two additional references in the Conclusions:
“It needs to be pointed out, that the investigated receptor polymorphisms could also play an important role in response to treatment with potential CCK receptor agonists as previously shown for other receptor polymorphisms, for example in liraglutide treatment in obese women with PCOS [30] as well as in other therapeutic fields [31].”
30. Jensterle M, Pirš B, Goričar K, Dolžan V, Janež A. Genetic variability in GLP-1 receptor is associated with inter-individual differences in weight lowering potential of liraglutide in obese women with PCOS: a pilot study. Eur J Clin Pharmacol 2015; 71: 817-24.
31. Murdaca G, Negrini S, Magnani O, Penza E, Pellecchio M, Puppo F. Impact of pharmacogenomics upon the therapeutic response to etanercept in psoriasis and psoriatic arthritis. Expert Opin Drug Saf 2017; 16: 1173-1179.

Reviewer 4:
Q: the authors should provide the actual numbers of patients included in each calculation for each parameter in all tables, and in Figure 1.
A: We have acknowledged in the Discussion as a limitation of our study that all the clinical data were not available for all patients, although they were managed in the same department at the UMC Ljubljana. In line with that we have included in the tables the number of missing data for each clinical and laboratory parameter and added the number of included patients also in the figure legend.

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Genetic variability in the cholecystokinin A receptor affects lipid profile and glucose tolerance in patients with polycystic ovary syndrome

Running head: *CCKAR* gene variability in PCOS

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Structured Abstract

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Material and methods: Our cross-sectional study included 168 patients with PCOS and 82 healthy female controls genotyped for polymorphisms in *CCKAR* (rs6448456 and rs1800857) and *CCKBR* (rs2929180, rs1800843, rs1042047 and rs1042048) genes.

Results: The investigated polymorphisms were not associated with anthropometric characteristics of patients with PCOS, however, among healthy controls carriers of at least one polymorphic CCKBR rs1800843 allele had bigger waist circumference (p=0.027) and more visceral fat (p=0.046). Among PCOS patients carriers of at least one polymorphic *CCKAR* rs6448456 C allele had significantly higher total blood cholesterol and LDL, and significantly lower blood glucose levels after 30, 60 and 90 minutes of the oral glucose tolerance test (all $p<0.05$). Healthy controls with at least one polymorphic CCKAR rs1800857 C allele were less likely to have high metabolic syndrome burden (p=0.029).

Conclusions: Genetic variability in *CCKAR* affects lipid profile and post-load glucose levels in patients with PCOS and is associated with metabolic sydrome burden in healthy young women. Further investigation of the role of genetic variability in *CCKAR* and *CCKBR* could contribute to development of individually tailored treatment strategies with CCK receptor agonists.

Keywords: cholecystokinin; *CCKAR; CCKBR;* PCOS; polymorphism

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine, reproductive and metabolic disorders in women of childbearing age [1]. The prevalence of PCOS in Europe varies widely, which could be attributable to different environmental and genetic factors [2]. Lifestyle modification in combination with metformin are considered as the main treatment strategy for metabolic phenotype of the syndrome. However, treatment goals including weight loss and normalization of glycaemic and lipid profile often remain unmet with the established approach and the need for the novel treatment options is growing [1, 3].

Cholecystokinin (CCK) is a digestive hormone, neuromodulator and neurotransmitter. Its biological actions are mediated by its binding and activation of cholecystokinin A receptor (CCKAR) and cholecystokinin B receptor (CCKBR) [4]. Binding of CCK via CCKAR increases postprandial satiety, increases secretion of digestive enzymes and endocrine hormones in the pancreas, acts as a growth factor on pancreatic beta cells, increases postprandial gallbladder emptying and delays gastric emptying. Binding of CCK or gastrin via CCKBR regulates pancreatic growth [4]. Furthermore, studies in mice with a homozygous *Cckbr* deletion suggest, that CCKBR could also increase postprandial satiety and improve glucose tolerance [5]. Therefore, CCK receptor agonists have a potential for the treatment of obesity, type 2 diabetes and also PCOS with high metabolic risk [6].

Many common functional polymorphisms are present in *CCKAR* and *CCKBR* genes that might affect signalling of endogenous CCK. Only a few studies addressing their genetic variability have been conducted up till now [7, 8]. A study of middle-aged and elderly adults showed that the homozygous carriers of two *CCKAR* promoter

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polymorphisms had increased fat content, higher insulin and higher leptin levels than wild-type and heterozygous individuals [7]. Another study reported association between *CCKBR* polymorphisms and antipsychotic induced weight gain in patients with schizophrenia [8].

According to our knowledge, the role of the *CCKAR* and *CCKBR* polymorphisms in patients with PCOS has not been addressed yet. We aimed to investigate genetic variability of *CCKAR* and *CCKBR* and their relationship with anthropometric and metabolic parameters in patients with PCOS.

Material and methods

Our cross-sectional study included 168 patients with PCOS treated at the outpatient clinics of Department of Endocrinology, Diabetes and Metabolic Diseases at the University Medical Centre (UMC) Ljubljana. Women were eligible for enrolment if they were more than 18 years old, premenopausal and diagnosed with phenotype A PCOS by Rotterdam criteria. Pregnant women and women who had diabetes were excluded. The study was approved by the Republic of Slovenia National Medical Ethics Committee and was carried out according to the Helsinki Declaration.

Anthropometric characteristics included were height, weight, waist circumference, BMI and visceral adipose tissue (VAT) area. Waist circumference was measured in a standing position midway between the lower costal margin and the iliac crest. BMI was calculated as the weight in kilograms divided by square of height in meters. Whole-body composition was assessed by a DXA (Discovery A; Hologic, Waltham, MA, USA) with the software provided by the manufacturer (QDR for Windows Version 12.5). Metabolic characteristics were obtained by drawing fasting blood and determining fasting blood glucose and insulin. Afterwards patients underwent a standard 75 g oral glucose tolerance

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test (OGTT). Glucose levels were determined using a standard glucose oxidase method (Beckman Coulter Glucose Analyzer, Beckman Coulter Inc., Brea, CA). Insulin levels were determined by immunoradiometric assay (Biosource Europe S.A., Nivelles, Belgium). Homeostasis model assessment ($HOMA_{IR}$) score calculation was applied as a measure for insulin resistance (IR) [9]. Values greater than 2.0 were considered as indicative of the presence of IR [10]. The World Health Organization diagnostic criteria of 2006 were used to determine basal baseline glycemia, impaired glucose tolerance and type 2 diabetes [11].

In addition, a healthy control group included 82 first year female students of the Nursing programme at the University of Ljubljana, Faculty of Health Sciences. The data on anthropometric characteristics included weight, BMI, waist circumference, visceral fat, fat mass and fat free mass, and the metabolic syndrome burden was defined as described by Šoštarič et al [12].

With regards to molecular genetic analysis, genomic DNA was extracted from venous blood using a FlexiGene DNA kit (Qiagen, Hilden, Germany). Polymorphisms in *CCKAR* and *CCKBR* were selected using LD TAG SNP Selection tool [13]. We considered only polymorphisms with a minor allele frequency (MAF) equal or greater than 0.05 in European population and a putative functional effect predicted using the SNP function prediction [13]. Linkage disequilibrium was checked using LDlink program for final selection of tag polymorphisms [14]. In total, two *CCKAR* tag polymorphisms (rs6448456, rs1800857) and four *CCKBR* tag polymorphisms (rs2929180, rs1800843, rs1042047, rs1042048) were genotyped using competitive allele specific KASPar assays according to the manufacturer's instructions (LGC, England). The selected polymorphisms, their predicted effect and genotype frequencies in our sample are shown in Supplementary Table I. The genotype frequencies of the *CCKBR* rs2929180 and the *CCKBR* rs1042047

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polymorphisms were not in Hardy-Weinberg equilibrium (HWE) in PCOS patients, thus *CCKBR* rs1042047 was excluded from further statistical analysis.

In the statistical analysis, the median and interquartile range were used to describe the central tendency and variability of continuous variables. Frequencies were used to describe the distribution of categorical variables. Deviation from HWE was evaluated using a standard chi-square test. If the genotype frequencies for a polymorphism were not in HWE, we used Fisher's exact test to compare the distribution of genotype frequencies of our sample with the expected distribution in the European population. In the subsequent analyses, a dominant genetic model was used. A nonparametric Mann-Whitney test was used to assess the associations of polymorphisms with continuous variables. Spearman's rho (ρ) was used to evaluate correlations between continuous variables. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed with IBM SPSS Statistics, version 21.0 (IBM Corporation, Armonk, NY, USA).

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Results

Anthropometric and metabolic characteristics of PCOS patients (N=168) are presented in Table I. The median total cholesterol and TAG were within the reference interval, while the median HDL was lower, and LDL was higher than the reference value. Insulin resistance was present in 71 out of 98 patients (72.4%) as assessed by $HOMA_{IR}$. Median fasting glucose and glucose concentration after 120 minutes and fasting insulin and insulin concentration after 120 minutes were within the reference interval. Nevertheless, 17 out of 142 patients (12.0%) had impaired fasting glucose and 37 out of 140 patients (26.4%) had impaired glucose tolerance. Several biochemical parameters were correlated with both increased BMI and increased waist circumference: TAG (ρ=0.211, P=0.015 and

ρ=0.285, P=0.002, respectively), $HOMA_{IR}$ (ρ=0.357, P<0.001 and ρ=0.261, P=0.011, respectively), fasting glucose (ρ=0.232, P=0.006 and ρ=0.187, P=0.034, respectively), glucose concentration after 120 minutes (ρ=0.291, P=0.001 and ρ=0.295, P=0.001, respectively) and fasting insulin (ρ=0.326, P=0.001 and ρ=0.219, P=0.033, respectively).

Anthropometric and metabolic characteristics of healthy controls (N=82) are presented in Table II. Controls were significantly younger compared to PCOS patients (P<0.001), had lower body weight, BMI and waist circumference (all P<0.001). Among healthy controls, only 2 (2.4%) had dyslipidemia and 29 (35.4%) had higher burden of metabolic syndrome.

Genotype distribution of the investigated polymorphisms is presented in Supplementary Table I. None of the investigated polymorphisms was associated with PCOS susceptibility in univariable analysis or after adjustment for age (Supplementary Table II).

None of the investigated polymorphisms in either *CCKAR* or *CCKBR* was associated with anthropometric characteristics of patients with PCOS as shown in Supplementary Tables III and IV, respectively. *CCKAR* polymorphisms were also not associated with anthropometric characteristics of healthy controls (Supplementary Table V). On the other hand, carriers of at least one polymorphic *CCKBR* rs1800843 allele among healthy controls had bigger waist circumference (p=0.027) and tended to have more visceral fat (p=0.046). Weight and BMI were also higher in these subjects, but the difference did not reach statistical significance (Supplementary Table VI).

The impact of the two *CCKAR* polymorphisms on metabolic characteristics of patients with PCOS is shown in Table III. Carriers of at least one polymorphic *CCKAR* rs6448456 C allele had significantly higher levels of total cholesterol (p=0.034) and LDL

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(p=0.036) compared to the wild-type genotype. Although there was no significant difference in fasting glucose concentration (p=0.253), carriers of at least one polymorphic *CCKAR* rs6448456 C allele had significantly lower blood glucose after 30 (p=0.022), 60 (p=0.001), and 90 (p=0.010) minutes of OGTT (Figure 1). After 120 minutes of OGTT carriers of at least one polymorphic *CCKAR* rs6448456 C allele still had a lower median blood glucose, but the difference was no longer statistically significant (p=0.092). No other significant associations between *CCKAR* polymorphisms and metabolic characteristics of patients with PCOS were observed.

The impact of *CCKBR* polymorphisms on metabolic characteristics of patients with PCOS is shown in Table IV. Carriers of at least one polymorphic *CCKBR* rs2929180 T allele had higher insulin concentration after 90 (p=0.004) minutes of OGTT. However, *CCKBR* rs2929180 T genotype was not associated with insulin concentration after 60 (p=0.152) or 120 (p=0.634) minutes of OGTT. No other significant associations between *CCKBR* polymorphisms and metabolic characteristics of patients with PCOS were detected.

Among healthy controls, only association with metabolic syndrome burden could be assessed (Table V). Carriers of at least one polymorphic *CCKAR* rs1800857 C allele were less likely to have high metabolic syndrome burden (p=0.029). No other significant associations between *CCKAR* or *CCKBR* polymorphisms and metabolic characteristics of healthy controls were observed.

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Discussion

We report for the first time on the impact of genetic variability in *CCKAR* and *CCKBR* on metabolic parameters in patients with PCOS. The main finding of our study was that the carriers of at least one polymorphic *CCKAR* rs6448456 C allele had a significantly

higher total cholesterol and LDL and a significantly lower blood glucose levels after 30, 60, and 90 minutes of OGTT.

First, we have assessed if the investigated *CCKAR* and *CCKBR* polymorphisms influence lipid profile in our group of patients with PCOS. A meta-analysis in 2011 showed that patients with PCOS have low HDL, increased triglycerides, increased LDL and increased non-HDL. Furthermore, these lipid parameters are worse in woman with PCOS than in healthy women regardless of BMI or ethnicity [15]. The guidelines from 2018 recommend that overweight and obese patients with PCOS, regardless of age, should have a fasting lipid profile at diagnosis and, thereafter, repeated measurements based on the presence of hyperlipidaemia and global cardiovascular disease risk [16]. In our study, carriers of at least one polymorphic *CCKAR* rs6448456 C allele had significantly higher total blood cholesterol and LDL than non-carriers. We have not found functional studies to explain our observations directly. The observed effect might be caused by an increased contraction of the gallbladder [4]. Our results could be in line with preclinical models, where intravenous administration of CCK in mice resulted in an increase in blood cholesterol and TAG due to an increased bile secretion mediated by CCK via CCK receptors and a subsequent reabsorption of the biliary lipids [17]. On the other hand, studies examining the effect of the *Cckar* gene deletion on cholesterol metabolism in animal models report conflicting data [17-19]. Another possible explanation for higher total blood cholesterol and LDL in carriers of at least one polymorphic *CCKAR* rs6448456 C allele may be the effect of CCK on secretion of pancreatic lipase [4]. It was shown that inhibition of lipase leads to decreased digestion of lipids resulting in lower total cholesterol and LDL levels [20]. Although we cannot unequivocally explain the mechanisms leading to higher total blood cholesterol and LDL levels in carriers of at least one polymorphic *CCKAR*

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rs6448456 C allele, our finding may be of clinical importance. Since CCK levels increase with aging [21] the risk for hypercholesterolemia in these patients is expected to increase over time and appropriate medical surveillance may be needed to prevent the related late complications.

Next, we have assessed if the investigated *CCKAR* and *CCKBR* polymorphisms influence glucose tolerance in our group of patients with PCOS. We have observed that carriers of at least one polymorphic *CCKAR* rs6448456 C allele had significantly lower blood glucose after 30, 60, and 90 minutes of OGTT. Since blood glucose is regulated by two processes, there are two possible explanations. The first explanation may be that delayed gastric emptying causes slower passage of glucose into the duodenum, leading to slower absorption and lower postprandial glucose. Our observation is in agreement with data reported in a study of eight healthy men who underwent OGTT, showing a delayed gastric emptying, a lower glucose peak and a lower insulin peak when they were administrated CCK as compared to when administrated saline. It is less likely that the observed effect might be due to faster glucose uptake into tissues in carriers of at least one polymorphic *CCKAR* rs6448456 C allele [22]. Namely, CCK secretion is mainly stimulated by proteins and fats, whereas glucose causes a significant but smaller rise of plasma CCK [23]. Moreover, a study on five healthy men showed that physiological concentrations of CCK potentiate amino acid-induced insulin secretion but not glucose-induced insulin secretion [24].

All investigated *CCKAR* and *CCKBR* polymorphisms are also common in general population (MAF between 9.5% and 38.2% according to dbSNP Allele Frequency Aggregator (ALFA) project). In our study, none of the investigated polymorphisms was associated with PCOS susceptibility. However, in young healthy women, *CCKBR*

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rs1800843 was associated with anthropometric characteristics and *CCKAR* rs1800857 with metabolic syndrome burden. Therefore, further studies are needed to assess the association of these polymorphisms with metabolic parameters also in other, non-PCOS patients.

One of the limitations of our study was that the genotype distributions were not in HWE for two out of six polymorphisms, namely *CCKBR* rs2929180 and *CCKBR* rs1042047. We decided to include the *CCKBR* rs2929180 polymorphism in further analysis because the distribution of genotype frequencies was not significantly different from the distribution reported for the European population, and there were no other polymorphisms in the respective gene region that could affect PCR amplification. However, we did not include the *CCKBR* rs1042047 polymorphism. Although the distribution of *CCKBR* rs1042047 genotype frequencies was not significantly different from the distribution in the dbSNP database, we found a common rs8192471 polymorphism only three nucleotides away from rs1042047 at the 5' end that could affect annealing and genotyping results. A similar conclusion was reported by other researchers [25].

Another limitation was that all the clinical data were not available for all patients, although they were managed in the same department at the UMC Ljubljana. Additionally, for healthy controls, not all anthropometric or metabolic parameters were measured. The association with dyslipidemia could not be evaluated among healthy controls, as only two subjects had altered lipid levels. Therefore, only association with metabolic syndrome burden was evaluated. As all the patients and controls were young adults, the risk that our data could be affected by treatments of other conditions was minimized. Furthermore, our study was not biased by genetic heterogeneity, as all subjects included in the study belonged to the Slovenian population, which is ethnically and genetically homogeneous [26].

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Conclusion

In conclusion, our data indicates interesting associations between the *CCKAR* rs6448456 polymorphism and the metabolic characteristics of patients with PCOS. Better understanding of the role of genetic variability in *CCKAR* and *CCKBR* may be of clinical importance for future development of treatment strategies with CCK receptor agonists, which have already been shown to enhance the weight lowering, appetite suppressing, and positive beta-cell actions of GLP-1 based drugs in preclinical models [27-29]. It needs to be pointed out, that the investigated receptor polymorphisms could also play an important role in response to treatment with potential CCK receptor agonists as previously shown for other receptor polymorphisms, for example in liraglutide treatment in obese women with PCOS [30] as well as in other therapeutic fields [31].

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Conflict of interest:

The authors declare no conflict of interest.

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Figure legends

Figure 1. Graph of blood glucose as a function of time (median with 95% confidence interval) in 83 PCOS patients with complete oral glucose tolerance test data. The grey curve indicates carriers of at least one polymorphic *CCKAR* rs6448456 allele. The black curve indicates carriers of two normal alleles.

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Table I. Characteristics of patients with polycystic ovary syndrome (N=168)

Characteristics	**Median (25–75%)**
Age (years)	30 (25–35.8)
Body weight (kg)	100 (85–111.3) [5]
BMI (kg/m^2)	35.8 (31.8–39.9) [6]
Waist circumference (cm)	112 (102–121.3) [31]
VAT area (cm^2)	156.5 (116.8–199.5) [74]
Total cholesterol (mmol/L)	4.7 (4.2–5.3) [33]
HDL (mmol/L)	1.2 (1–1.4) [33]
LDL (mmol/L)	2.9 (2.4–3.4) [34]
TAG (mmol/L)	1.4 (1–1.8) [33]
HOMA$_{IR}$ (/)	2.8 (1.8–5) [70]
Glucose 0 min OGTT (mmol/L)	5.2 (4.8–5.6) [26]
Glucose 30 min OGTT (mmol/L)	8.3 (6.9–9.4) [79]
Glucose 60 min OGTT (mmol/L)	8.3 (6.8–9.8) [80]
Glucose 90 min OGTT (mmol/L)	7.6 (5.9–9) [82]
Glucose 120 min OGTT (mmol/L)	6.6 (5.5–7.9) [28]
Insulin 0 min OGTT (mU/L)	12.4 (7.5– 20) [70]
Insulin 30 min OGTT (mU/L)	71.3 (45.1–104.8) [84]
Insulin 60 min OGTT (mU/L)	97.5 (63.6–132.3) [84]
Insulin 90 min OGTT (mU/L)	87.9 (61.9–121.5) [84]
Insulin 120 min OGTT (mU/L)	78.5 (52.8–124.3) [76]

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BMI – body mass index; VAT – visceral adipose tissue; HDL – high density lipoproteins; LDL – low density lipoproteins; TAG – triacylglycerols; $HOMA_{IR}$ – homeostatic model assessment for insulin resistance; OGTT – oral glucose tolerance test; [] – missing data

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Table II. Characteristics of healthy controls (N=82).

Characteristics	**Median (25–75%)**
Age (years)	20 (20-22)
Body weight (kg)	62.2 (55.5-72.2)
BMI (kg/m^2)	22.4 (20.2-25.0)
Waist circumference (cm)	73 (66.8-78)
Visceral fat (arbitrary units)	3 (3-4)
Fat mass (%)	32.3 (26.3-37.1)
Fat free mass (%)	28.5 (27-30.6)

BMI – body mass index

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Table III. *CCKAR* polymorphisms and metabolic characteristics of patients with polycystic ovary syndrome (N=168).

Characteristics	**Genotype***	*CCKAR* rs6448456		*CCKAR* rs1800857	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**
Total cholesterol (mmol/L) [33]	X/X	4.6 (4.2–5.1)	**0.034**	4.7 (4.2–5.3)	0.880
	X/x+x/x	5.1 (4.4–5.6)		4.8 (4.2–5.3)	
HDL (mmol/L) [33]	X/X	1.2 (1–1.4)	0.749	1.2 (1.1–1.4)	0.053
	X/x+x/x	1.2 (1–1.4)		1.2 (1–1.3)	
LDL (mmol/L) [34]	X/X	2.8 (2.3–3.4)	**0.036**	2.9 (2.4–3.4)	0.944
	X/x+x/x	3.2 (2.5–3.6)		2.9 (2.4–3.4)	
TAG (mmol/L) [33]	X/X	1.4 (1.1–2)	0.373	1.3 (1–1.9)	0.282
	X/x+x/x	1.4 (1–1.7)		1.6 (1.2–1.8)	
$HOMA_{IR}$ (/) [70]	X/X	2.7 (1.7–4.6)	0.180	2.7 (1.7–5.1)	0.503
	X/x+x/x	3.1 (2.5–5.6)		3.2 (2–4.9)	
Glucose 0 min OGTT (mmol/L) [26]	X/X	5.2 (4.8–5.7)	0.253	5.1 (4.8–5.5)	0.219
	X/x+x/x	5.1 (4.9–5.4)		5.2 (4.9–5.7)	

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Glucose 30 min OGTT (mmol/L) [79]	X/X	8.7 (7.3–9.5)	**0.022**	8 (6.8–9.3)	0.236
	X/x+x/x	7.7 (6.5–8.5)		8.7 (7.1–10.3)	
Glucose 60 min OGTT (mmol/L) [80]	X/X	9.3 (7.4–10.7)	**0.001**	7.8 (6.6–9.6)	0.059
	X/x+x/x	7.1 (5.9–8.7)		9 (7.3–11.2)	
Glucose 90 min OGTT (mmol/L) [82]	X/X	7.9 (6.3–9.5)	**0.010**	7.4 (5.9–8.8)	0.204
	X/x+x/x	6.7 (5.6–8.1)		7.9 (6.4–9.6)	
Glucose 120 min OGTT (mmol/L) [28]	X/X	6.9 (5.6–8.2)	0.092	6.6 (5.4–7.8)	0.488
	X/x+x/x	6.3 (5.4–7.4)		6.5 (5.8–8)	
Insulin 0 min OGTT (mU/L) [70]	X/X	11.5 (7.4–19.7)	0.141	12.4 (7.1–20.2)	0.839
	X/x+x/x	15.1 (11–21.8)		13 (8.8–19.5)	
Insulin 30 min OGTT (mU/L) [84]	X/X	70.3 (43.2–106.3)	0.468	77.4 (50.6–110.8)	0.324
	X/x+x/x	78.5 (52.3–102.7)		65.7 (40.9–102.9)	
Insulin 60 min OGTT (mU/L) [84]	X/X	98 (63.5–136.3)	0.681	86.4 (63.2–120.5)	0.302
	X/x+x/x	89.5 (63.9–127.3)		100.7 (67.3–146)	
Insulin 90 min OGTT (mU/L) [84]	X/X	82.3 (60.5–145)	0.873	88.2 (58.3–125)	0.730

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	X/x+x/x	93.6 (67.7–118)		87.9 (69.6–119.5)	
Insulin 120 min OGTT (mU/L)	X/X	79.3 (53.3–127)	0.730	78 (51.8–125)	0.981
[76]	X/x+x/x	74.9 (52.3–118.3)		79.5 (56.1–121.5)	

* X – major (common) allele, x – minor (variant) allele

HDL – high density lipoproteins; LDL – low density lipoproteins; TAG – triacylglycerols; $HOMA_{IR}$ – homeostatic model assessment for insulin resistance; OGTT – oral glucose tolerance test; [] – number of missing data

Statistically significant p values are printed in bold.

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Table IV. *CCKBR* polymorphisms and metabolic characteristics of patients with polycystic ovary syndrome (N=168).

Characteristics	**Genotype***	*CCKBR* rs2929180		*CCKBR* rs1800843		*CCKBR* rs1042048	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**	**Median (25–75%)**	**p**
Total cholesterol (mmol/L) [33]	X/X	4.8 (4.3–5.4)	0.226	4.7 (4.2–5.5)	0.805	4.7 (4.2–5.4)	0.679
	X/x+x/x	4.6 (4.1–5.3)		4.7 (4.2–5.2)		4.7 (4.2–5.3)	
HDL (mmol/L) [33]	X/X	1.2 (1–1.4)	0.358	1.2 (1.1–1.4)	0.998	1.2 (1–1.3)	0.332
	X/x+x/x	1.2 (1–1.3)		1.2 (1–1.4)		1.2 (1–1.4)	
LDL (mmol/L) [34]	X/X	2.9 (2.4–3.4)	0.471	2.9 (2.4–3.5)	0.962	2.9 (2.4–3.5)	0.484
	X/x+x/x	2.8 (2.3–3.6)		2.8 (2.4–3.4)		2.8 (2.3–3.4)	
TAG (mmol/L) [33]	X/X	1.5 (1.1–2)	0.083	1.5 (1.1–1.8)	0.331	1.4 (1.2–1.8)	0.979
	X/x+x/x	1.2 (0.9–1.7)		1.4 (0.9–1.9)		1.5 (1–2)	
$HOMA_{IR}$ (/) [70]	X/X	3.1 (1.9–5.1)	0.171	2.8 (1.8–4.6)	0.838	3.2 (2.3–5.1)	0.290
	X/x+x/x	2.4 (1.5–4.6)		2.9 (1.7–5.6)		2.7 (1.7–4.9)	
Glucose 0 min OGTT (mmol/L) [26]	X/X	5.1 (4.8–5.6)	0.980	5.2 (4.8–5.5)	0.784	5.2 (4.8–5.6)	0.917
	X/x+x/x	5.2 (4.9–5.6)		5.2 (4.9–5.6)		5.2 (4.9–5.6)	

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Glucose 30 min OGTT (mmol/L) [79]	X/X	8.1 (6.9–9.4)	0.542	8.2 (6.8–9.4)	0.513	8.3 (6.8–10)	0.990
	X/x+x/x	8.7 (7–9.4)		8.4 (7.3–9.4)		8.1 (7.1–9.3)	
Glucose 60 min OGTT (mmol/L) [80]	X/X	8.1 (6.6–9.8)	0.199	8.3 (6.6–9.8)	0.909	8.6 (6.2–11.1)	0.571
	X/x+x/x	9.2 (7.3–10.7)		8.7 (7.1–9.8)		7.8 (7–9.5)	
Glucose 90 min OGTT (mmol/L) [82]	X/X	7.1 (5.8–8.8)	0.106	7.4 (5.8–9.1)	0.704	7.7 (5.7–9.6)	0.826
	X/x+x/x	8.1 (6.8–9.5)		7.9 (6.4–8.9)		7.6 (6.5–8.7)	
Glucose 120 min OGTT (mmol/L) [28]	X/X	6.6 (5.6–7.9)	0.661	6.6 (5.4–7.8)	0.695	6.3 (5.4–7.9)	0.594
	X/x+x/x	6.4 (5.3–7.8)		6.6 (5.6–7.9)		6.8 (5.6–7.8)	
Insulin 0 min OGTT (mU/L) [70]	X/X	13 (8.3–20.2)	0.185	12.4 (7.6–19.1)	0.655	13.3 (9.6–21.1)	0.321
	X/x+x/x	10.4 (6.3–19.8)		13.5 (7.3–23)		11.8 (6.8–19.7)	
Insulin 30 min OGTT (mU/L) [84]	X/X	71.2 (45.9–99.4)	0.831	69.3 (42.7–98.3)	0.067	72.2 (44.8–106.3)	0.834
	X/x+x/x	80.5 (38.7–115.5)		87.7 (53–124.5)		71.4 (44.4–105.5)	
Insulin 60 min OGTT (mU/L) [84]	X/X	87.7 (62–115.5)	0.152	90.7 (68.4–129)	0.670	99.2 (66.8–133.8)	0.397
	X/x+x/x	117.5 (64.7–145.5)		97.9 (58.1–145)		88.2 (52.3–131)	
Insulin 90 min OGTT (mU/L) [84]	X/X	80.1 (58.3–113.5)	**0.004**	82.5 (60.6–117.5)	0.145	92.3 (70.6–123)	0.461

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	X/x+x/x	118 (83.4–168.8)		104 (66.4–183.5)		81.8 (57.8–124)	
Insulin 120 min OGTT (mU/L) [76]	X/X	79.5 (51.4–119.5)	0.634	74.9 (51.7–107)	0.121	86.7 (65.3–128)	0.096
	X/x+x/x	77.8 (57.8–142)		88.7 (61.5–148)		66.9 (46–118)	

* X – major (common) allele, x – minor (variant) allele

HDL – high density lipoproteins; LDL – low density lipoproteins; TAG – triacylglycerols; $HOMA_{IR}$ – homeostatic model assessment for insulin resistance; OGTT – oral glucose tolerance test; [] – number of missing data

Statistically significant p values are printed in bold.

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Table V. Association of selected *CCKAR* and *CCKBR* polymorphisms with metabolic syndrome burden in healthy controls (N=82).

SNP	Genotype	Low burden N (%)	High burden N (%)	OR (95% CI)	p
***CCKAR* rs6448456**	G/G	38 (71.7)	16 (55.2)	Reference	
	G/C+C/C	15 (28.3)	13 (44.8)	2.06 (0.80-5.30)	0.134
***CCKAR* rs1800857**	T/T	33 (62.3)	25 (86.2)	Reference	
	T/C+C/C	20 (37.7)	4 (13.8)	0.26 (0.08-0.87)	**0.029**
***CCKBR* rs2929180**	G/G	37 (69.8)	18 (62.1)	Reference	
	G/T+T/T	16 (30.2)	11 (37.9)	1.41 (0.55-3.66)	0.477
***CCKBR* rs1800843**	C/C	36 (67.9)	19 (65.5)	Reference	
	C/A+A/A	17 (32.1)	10 (34.5)	1.12 (0.43-2.91)	0.825
***CCKBR* rs1042048**	A/A	24 (45.3)	9 (31.0)	Reference	
	A/G+G/G	29 (54.7)	20 (69.0)	1.84 (0.71-4.78)	0.211

OR – odds ratio; CI – confidence interval
Statistically significant p values are printed in bold.

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Supplementary Table I. Genotype frequencies and predicted functional effect of selected *CCKAR* and *CCKBR* polymorphisms

			Healthy controls (N=82)			**PCOS patients (N=168)**		
SNP	**Predicted effect**	**Genotype**	**N (%)**	**MAF**	**pHWE**	**N (%)**	**MAF**	**pHWE**
***CCKAR* rs6448456**	binding of	G/G	54 (65.9)	0.189	0.960	114 (67.9)	0.182	0.448
c.-85G>C	transcription factors	G/C	25 (30.5)			47 (28.0)		
5'UTR	and intron splicing	C/C	3 (3.7)			7 (4.2)		
***CCKAR* rs1800857**	binding of	T/T	58 (70.7)	0.156	0.121	115 (68.5)	0.167	0.355
c.113-5T>C	transcription factors	T/C	24 (29.3)			50 (29.8)		
intron		C/C	0 (0.0)			3 (1.8)		
***CCKBR* rs2929180**	binding of	G/G	55 (67.1)	0.177	0.669	121 (72.0)	0.170	**0.005**
c.-51G>T	transcription factors	G/T	25 (30.5)			37 (22.0)		
5'UTR		T/T	2 (2.4)			10 (6.0)		
***CCKBR* rs1800843**	intron splicing	C/C	55 (67.1)	0.171	0.278	108 (65.1) [2]	0.187	0.361
c.559+32C>A		C/A	26 (31.7)			54 (32.5)		
intron		A/A	1 (1.2)			4 (2.4)		

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***CCKBR* rs1042047** c.*409A>C 3'UTR	miRNA binding site	A/A	35 (42.7)	0.378	0.123	81 (48.5) [1]	0.347	**<0.001**
		A/C	32 (39.0)			56 (33.5)		
		C/C	15 (18.3)			30 (18.0)		
***CCKBR* rs1042048** c.*437A>G 3'UTR	miRNA binding site	A/A	33 (40.2)	0.341	0.209	80 (48.2) [2]	0.322	0.182
		A/G	42 (51.2)			65 (39.2)		
		G/G	7 (8.5)			21 (12.7)		

SNP – single nucleotide polymorphism; 5'UTR – 5'-untrancribed region; 3'UTR – 3'-untranslated region; MAF – minor allele frequency; pHWE – HardyWeinberg equilibrium; [] – number of missing data

Statistically significant p values are printed in bold.

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Supplementary Table II. Association of selected *CCKAR* and *CCKBR* polymorphisms with PCOS susceptibility.

Polymorphism	Genotype	Controls N (%)	PCOS N (%)	OR (95% CI)	p	OR (95% CI)$_{adj}$	p$_{adj}$
***CCKAR* rs6448456**	G/G	54 (65.9)	114 (67.9)	Reference		Reference	
	G/C+C/C	28 (34.1)	54 (32.1)	0.91 (0.52-1.60)	0.751	1.15 (0.60-2.22)	0.680
***CCKAR* rs1800857**	T/T	58 (70.7)	115 (68.5)	Reference		Reference	
	T/C+C/C	24 (29.3)	53 (31.5)	1.11 (0.63-1.98)	0.714	1.07 (0.54-2.11)	0.843
***CCKBR* rs2929180**	G/G	55 (67.1)	121 (72.0)	Reference		Reference	
	G/T+T/T	27 (32.9)	47 (28.0)	0.79 (0.45-1.40)	0.421	0.82 (0.42-1.62)	0.574
***CCKBR* rs1800843**	C/C	55 (67.1)	108 (65.1)	Reference		Reference	
	C/A+A/A	27 (32.9)	58 (34.9)	1.09 (0.63-1.92)	0.753	1.42 (0.74-2.75)	0.295
***CCKBR* rs1042048**	A/A	33 (40.2)	80 (48.2)	Reference		Reference	
	A/G+G/G	49 (59.8)	86 (51.82)	0.72 (0.42-1.24)	0.238	0.93 (0.50-1.75)	0.825

PCOS – polycystic ovary syndrome; OR – odds ratio; CI – confidence interval; adj – adjusted for age

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Supplementary Table III. *CCKAR* polymorphisms and anthropometric characteristics of patients with polycystic ovary syndrome (N=168).

Characteristics	**Genotype***	*CCKAR* rs6448456		*CCKAR* rs1800857	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**
Body weight (kg) [5]	X/X	100.9 (86.9–112.3)	0.245	99.7 (85–110.8)	0.410
	X/x+x/x	97.3 (82.6–107.5)		101 (84.3–111.4)	
BMI (kg/m^2) [6]	X/X	36.2 (32–40.6)	0.143	35.8 (31.3–39.7)	0.362
	X/x+x/x	34.3 (30.9–38.5)		35.5 (32–41.1)	
Waist circumference (cm) [31]	X/X	112 (102–123)	0.591	110 (100.5–121.3)	0.303
	X/x+x/x	111 (98.8–121)		113.5 (102.3–122.3)	
VAT area (cm^2) [74]	X/X	159 (119–205)	0.285	146.5 (108.8–197.8)	0.244
	X/x+x/x	145 (113–179.5)		167.5 (127–201)	

* X – major (common) allele, x – minor (variant) allele

BMI – body mass index; VAT – visceral adipose tissue; [] – number of missing data

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Supplementary Table IV. *CCKBR* polymorphisms and anthropometric characteristics of patients with polycystic ovary syndrome (N=168).

Characteristics	**Genotype***	*CCKBR* rs2929180		*CCKBR* rs1800843		*CCKBR* rs1042048	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**	**Median (25–75%)**	**p**
Body weight (kg) [5]	X/X	100 (85–111.6)	0.933	101 (84.5–111.7)	0.503	100 (86.6–110.1)	0.985
	X/x+x/x	101 (84.9–110.9)		96.2 (85.2–111.1)		100.9 (85–114)	
BMI (kg/m^2) [6]	X/X	35.7 (31.9–40.3)	0.704	35.9 (31.5–39.9)	0.836	36.3 (32.4–39.9)	0.781
	X/x+x/x	36.6 (31.5–39.1)		35.8 (31.8–40.8)		35.6 (30.9–40.3)	
Waist circumference (cm) [31]	X/X	110.3 (101.8–123)	0.917	110 (101–122)	0.412	112 (105.1–122.9)	0.371
	X/x+x/x	114 (102–121)		113.5 (103.8–121)		109.5 (100–121)	
VAT area (cm^2) [74]	X/X	148 (115.5–203)	0.402	150 (116–186.5)	0.510	165 (135–194)	0.329
	X/x+x/x	165 (132.5–189)		157.5 (118.5–208)		147.5 (114–201)	

* X – major (common) allele, x – minor (variant) allele

BMI – body mass index; VAT – visceral adipose tissue; [] – number of missing data

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Supplementary Table V. *CCKAR* polymorphisms and anthropometric characteristics of healthy controls (N=82).

Characteristics	**Genotype***	*CCKAR* rs6448456		*CCKAR* rs1800857	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**
Body weight (kg)	X/X	61.4 (55.2-70.8)	0.214	62.2 (55.2-72.2)	0.819
	X/x+x/x	64.1 (57.7-72.3)		61.6 (56.7-73)	
BMI (kg/m^2)	X/X	22.1 (19.8-24.6)	0.205	22.5 (20.2-25)	0.695
	X/x+x/x	22.7 (20.8-25.4)		22.2 (20.1-25)	
Waist circumference (cm)	X/X	73 (66-77.3)	0.419	73 (66.8-79)	0.874
	X/x+x/x	73.5 (67.5-80.5)		73 (66.3-78)	
Visceral fat (arbitrary units)	X/X	3 (2-4)	0.217	3.5 (3-4.3)	0.314
	X/x+x/x	3.5 (3-5)		3 (2.3-4)	
Fat mass (%)	X/X	30.4 (25.8-35.9)	0.172	32.6 (26.8-37.1)	0.650
	X/x+x/x	33.3 (27.6-37.9)		30.4 (26.1-37.1)	
Fat free mass (%)	X/X	28.8 (27.5-30.8)	0.148	28.3 (27.1-30)	0.551
	X/x+x/x	28.1 (25.9-29.5)		28.8 (26.6-30.9)	

* X – major (common) allele, x – minor (variant) allele

BMI – body mass index

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Supplementary Table VI. *CCKBR* polymorphisms and anthropometric characteristics of healthy controls (N=82).

Characteristics	**Genotype***	*CCKBR* rs2929180		*CCKBR* rs1800843		*CCKBR* rs1042048	
		Median (25–75%)	**p**	**Median (25–75%)**	**p**	**Median (25–75%)**	**p**
Body weight (kg)	X/X	62.1 (55-70.4)	0.329	60.8 (55-70.3)	0.077	62.1 (54.3-74.5)	0.925
	X/x+x/x	63.6 (57-76.7)		65.7 (58-77.1)		62.2 (56.7-71.3)	
BMI (kg/m^2)	X/X	22.1 (19.6-24.8)	0.135	22 (19.6-24.9)	0.071	22.3 (19.4-25.5)	0.828
	X/x+x/x	22.4 (21.1-27.1)		23.5 (21.6-25.2)		22.4 (20.8-24.9)	
Waist circumference (cm)	X/X	71 (66-78)	0.222	69 (66-78)	**0.027**	73 (66.5-80)	0.809
	X/x+x/x	73 (69-82)		74 (71-78)		73 (66.5-78)	
Visceral fat (arbitrary units)	X/X	3 (2-4)	0.143	3 (2-4)	**0.046**	4 (2.5-5)	0.502
	X/x+x/x	3 (3-5)		4 (3-5)		3 (3-4)	
Fat mass (%)	X/X	31.6 (26.3-35.6)	0.319	30.2 (25.5-37.1)	0.109	32.7 (25.5-37.1)	0.755
	X/x+x/x	32.8 (26.3-38.2)		33.9 (30.5-36.6)		32.2 (26.7-36.9)	
Fat free mass (%)	X/X	28.5 (27.5-30.4)	0.453	28.9 (27.1-30.8)	0.364	28.3 (27.2-30.8)	0.992
	X/x+x/x	28.3 (25.5-31.5)		28.3 (26.7-29.3)		28.7 (26.7-30.3)	

* X – major (common) allele, x – minor (variant) allele

BMI – body mass index

Statistically significant p values are printed in bold.

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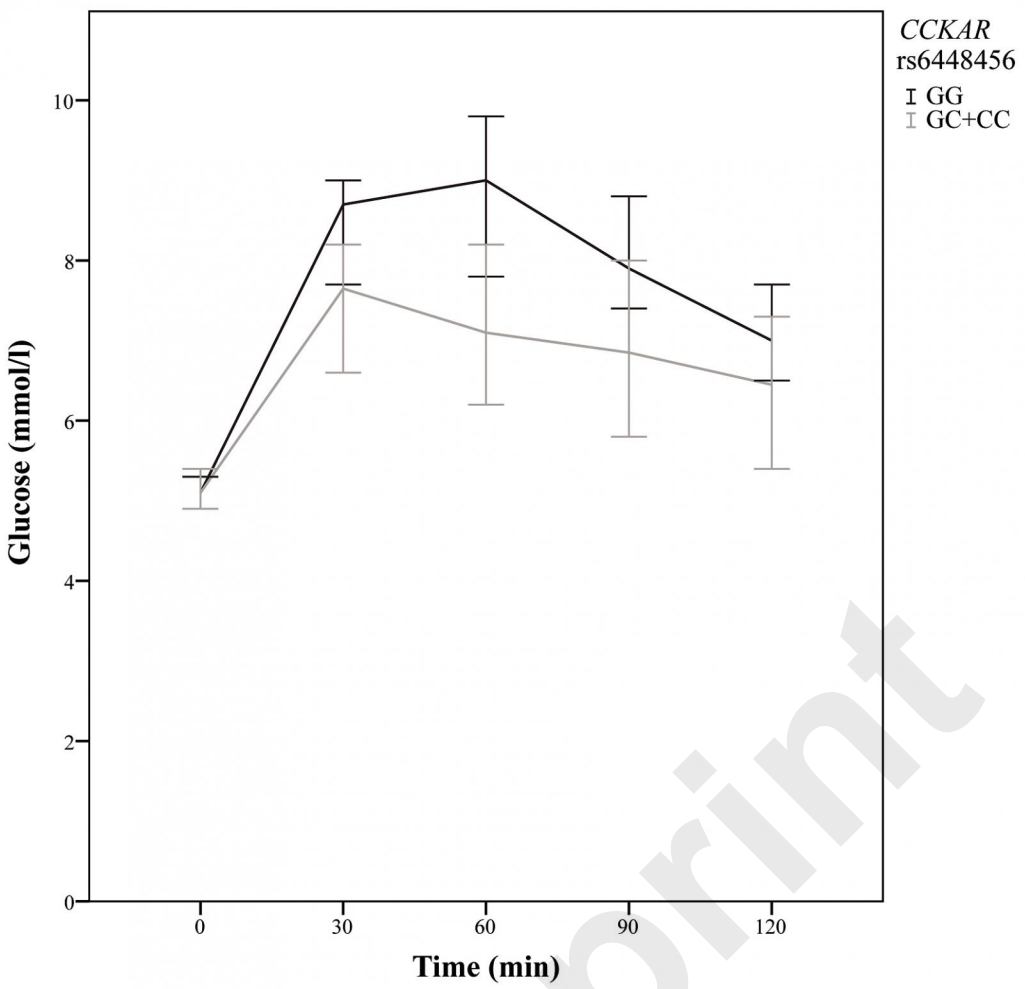

Figure 1. Graph of blood glucose as a function of time (median with 95% confidence interval). The grey curve indicates carriers of at least one polymorphic CCKAR rs6448456 allele. The black curve indicates carriers of two normal alleles.

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