1   **Title**

2   **Genome-wide methylation profiling in granulosa lutein cells of women with polycystic**

3   **ovary syndrome (PCOS)**

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Abstract

Polycystic Ovary Syndrome (PCOS) is the most common endocrine disorder amongst women of reproductive age, whose aetiology remains unclear. To improve our understanding of the molecular mechanisms underlying the disease, we conducted a genome-wide DNA methylation profiling in granulosa lutein cells collected from 16 women suffering from PCOS, in comparison to 16 healthy controls. Samples were collected by follicular aspiration during routine egg collection for IVF treatment. Study groups were matched for age and BMI, did not suffer from other disease and were not taking confounding medication.

 Comparing women with polycystic versus normal ovarian morphology, after correcting for multiple comparisons, we identified 106 differentially methylated CpG sites with p-values $<5.8 \times 10^{-8}$ that were associated with 88 genes, several of which, are known to relate either to PCOS or to ovarian function. Replication and validation of the experiment was done using pyrosequencing to analyse six of the identified differentially methylated sites. Pathway analysis indicated potential disruption in canonical pathways and gene networks that are, amongst other, associated with cancer, cardiogenesis, Hedgehog signalling and immune response. In conclusion, these novel findings indicate that women with PCOS display epigenetic changes in ovarian granulosa cells that may be associated with the heterogeneity of the disorder.

1. Introduction

Polycystic Ovary Syndrome (PCOS) is a common, heterogeneous endocrine disorder with an estimated prevalence up to 15% amongst women of reproductive age, depending on the diagnostic criteria used (Azziz *et al.,* 2004, Franks, 1995, March *et al.,* 2010, Sirmans and Pate, 2013). It comprises metabolic and reproductive disturbances, whereas environmental influences, such as diet, are known to affect the phenotype. Its main biochemical

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1 characteristic is the hypersecretion of androgens, predominantly of ovarian origin that is

2 associated with, and may be causally related to, infertility (anovulation, menstrual

3 irregularities), hirsutism (hair excess), psychological distress (anxiety, depression) and

4 metabolic defects (obesity, insulin resistance). Women with PCOS have an increased risk of

5 developing type 2 diabetes and cardiovascular disease later in life (Diamanti-Kandarakis and

6 Dunaif, 2012, Jayasena and Franks, 2014, Mani *et al.,* 2013, Qu *et al.,* 2012).

7 The aetiology of PCOS remains unclear. There is, however, evidence for genetic

8 predisposition with familial clustering of cases as well as genetic variants of endocrine and

9 metabolic markers (Barber and Franks, 2013, Chen *et al.,* 2011, Day *et al.,* 2015, Franks *et*

10 *al.,* 1997, Franks *et al.,* 2008, Hayes *et al.,* 2015, Shi *et al.,* 2012, Vink *et al.,* 2006). It has

11 been proposed that PCOS originates in early (possibly fetal) life due to "programming" by

12 exposure to excessive androgen production. (Abbott *et al.,* 2005, Abbott *et al.,* 2002, Franks

13 and Berga, 2012, Li and Huang, 2008, Xita and Tsatsoulis, 2006).

14 Results of protein and expression profiling experiments in adults support the view that there

15 is an important contribution of androgen-dependent genes to the aetiology of PCOS (Adams

16 *et al.,* 2016, Coskun *et al.,* 2013, Insenser and Escobar-Morreale, 2011, Li *et al.,* 2016, Lv *et*

17 *al.,* 2017). There are however very few studies regarding the epigenetic changes associated

18 with PCOS development, with only a handful of genome-wide studies that were conducted

19 mainly on whole blood, or ovarian tissue (Table S1).

20 The rationale of our study is based on the hypothesis that PCOS can be explained by an

21 integrated epigenetic model, whereby environmental factors modify the effect of

22 susceptibility genes and therefore, influence the clinical and biochemical heterogeneity that

23 is characteristic of the syndrome during adult life. In this investigation, we report results from

24 a PCOS case-control, genome-wide methylation study using DNA from granulosa lutein cells

25 (GLCs) that are known to be androgen responsive, with the aim to advance our

26 understanding of the molecular mechanisms underlying the disease, to provide a novel

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1 insight into the role of epigenetic programming in PCOS and ultimately to improve its

2 diagnosis and treatment.

3

2. Materials and Methods

2.1. Patient Recruitment and Sample Collection

6 GLC samples from consecutive subjects with or without PCOS, were collected from the

7 Hammersmith IVF clinic (Wolfson Fertility Centre, Hammersmith Hospital) by follicular

8 aspiration of mature oocytes (>14mm diameter on the day of HCG administration), during

9 routine egg collection.

10 All participants provided informed consent. PCOS was diagnosed according to the

11 Rotterdam Consensus criteria (Rotterdam, 2004). Sample collection was approved by the

12 National Research Ethics Service (NRES) (Hammersmith & Queen Charlotte's Chelsea:

13 08/H0707/152).

14 The array-based analysis cohort comprised 32 samples, 16 PCOS women with

15 oligomenorrhea and 16 healthy women (controls), who received IVF treatment due to male

16 infertility factors. All participants were of similar age and BMI, did not suffer either currently,

17 or in the past from other disease and were not taking Metformin medication for insulin

18 resistance (Table 1).

19 The validation cohort using pyrosequencing consisted of two PCOS subgroups, with (n=27)

20 and without (n=15) oligomenorrhea and healthy controls undergoing IVF treatment due to

21 male infertility factor and/or physical blockage (n=43, 2 of them apart from male infertility,

22 also had salpingectomies). Samples were overlapping with the samples used for the

23 genome-wide analysis. All extra samples were from subjects who had same clinical

24 characteristics as those included in the array-based study (Table 1).

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2.2. Genomic DNA Isolation and Quantitation

GLCs were isolated by density gradient centrifugation using Percoll (GE Healthcare Life Sciences, UK). Genomic DNA was extracted using the QIAGEN DNeasy Blood and tissue extraction kit according to the manufacturer's instructions (QIAGEN Ltd).

Following extraction, DNA was quantified by measuring absorbance on a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) and fluorescence intensity using the Quant-iT PicoGreen dsDNA Assay (Life Technologies Limited) on a PHERAstar FS multi-mode reader (BMG LABTECH Ltd). Both quantitation methods were considered.

2.3. DNA Methylation Microarray

Genome-wide DNA methylation profiling was generated using the Infinium MethylationEPIC BeadChip array (Illumina, San Diego, CA, USA). 500ng of DNA were bisulfite converted using the EZ-96 DNA Methylation™ Kit (Zymo Research Corporation, Irvine, CA), fragmented and hybridised on the BeadChip. Signal intensities were extracted using the Illumina iScan Reader (Illumina, San Diego, CA, USA).

Hybridization, scanning and raw data processing were performed by the Oxford Genomics Centre facilities (Wellcome Trust Centre for Human Genetics, Oxford), according to Illumina's protocol (https://support.illumina.com).

2.4. Quality Control and Data Pre-processing

Raw data files and genomic annotation from the EPIC beadchip array were provided by the Oxford Genomics Centre service. Raw intensities (.idat) were retrieved and pre-processed using the Bioconductor package minfi (version 1.18.6) in R (Fortin *et al.,* 2017).

1 All samples passed the initial quality assessment, with an intensity detection p-value $< 10^{-16}$

2 and a sample call rate > 98%. Intensity values were quantile normalised using *limma* (Smyth

3 *et al.,* 2005) and converted to β-values, reported as a score ranging from 0 (non-methylated)

4 to 1 (completely methylated).

5 Global correlation patterns and sample relationships for the detection of biological clustering

6 and outliers were assessed by Principal Component Analysis (PCA). We included all

7 samples in the study and all markers that had no missing data (n=850,514, *prcomp* function

8 in R with default settings). PCA variance was 9.25% and 7.55% for PC1 and PC2

9 respectively. Linear models regressing the case-control status against the first two principal

10 components indicated a separation between the two groups by PC1 (p=0.04) and PC2

11 (p=0.004).

12 PCA clustering indicated the presence of two potential outliers (1 PCOS; red colour and 1

13 control; black colour, Supplementary Fig. S1). However, comparing data of linear regression

14 analysis with and without the two samples, we identified 62 overlapping hits within the cut-off

15 threshold of $p < 5.8 \times 10^{-8}$, whereas 44 hits (out of 106) had p-values very close to the ones

16 identified including all samples, ranging from 5.0×10^{-08} to 2.9×10^{-07}. The remaining 10 hits,

17 identified when we excluded the two outliers, were not associated with any genes, and

18 mainly had intergenic locations. Since neither sample deviated from the clinical phenotype

19 that characterises the two study groups to which they belonged, they were considered not as

20 biological, but rather as technical outliers, and were included in the analysis (Supplementary

21 Table S2).

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23 *2.5. Statistical analysis*

24 Downstream statistical analysis of the data was performed using the CPACOR

25 (incorporating Control Probe Adjustment and reduction of global CORrelation) methylation

26 analysis pipeline (Lehne *et al.,* 2015) in R (http://cran.r-project.org, version 3.3). The analysis

1 was corrected for multiple comparisons (Bonferroni correction for multiple testing;

2 $0.05/853,307 = 5.8 \times 10^{-8}$).

3 To improve data quality and adjust for technical variations introduced by the use of two

4 probe types (I and II) with distinct differences, data were normalised as raw data (beta

5 values) without pre-processing, and using Illumina Genome Studio (reverse engineered and

6 implemented in minfi), Subset-quantile within array (SWAN), Quantile, Functional (FunNorm)

7 and Noob normalisation methods (Fortin *et al.*, 2017) (Supplementary Table S2). The

8 highest correlations between paired methylation measurements were observed after quantile

9 normalisation. Therefore, we based our analysis on a general linear regression model with

10 quantile normalised data.

11 The distribution of p-values under the null hypothesis was determined by randomly re-

12 assigning a case-control status to all 32 samples of the study and performing a linear

13 regression analysis for each marker, using normalised data, with and without adjusting for

14 the two control probes. 1000 permutations were performed each time, to obtain 2 x 1000 p-

15 value sets under the assumption of no association.

16 For comparison of our data with the Xu *et al.* study (the only previously published study

17 using granulosa-lutein cells), we considered a "hit" in the Xu *et al.* study as replicated if there

18 was directional consistency between the effect sizes, with $p < 0.05$. We then compared how

19 many hits were replicated versus the total number of published hits, in each category, using

20 a one-sided binom.test() with unadjusted cut-off $p = 0.05$.

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22 *2.6. Pyrosequencing*

23 Validation of the selected gene-associated CpG sites was performed using the Q96 MD

24 Pyrosequencing platform (QIAGEN Ltd). Primers for the pyrosequencing assays were

25 designed using the Pyromark Assay Design Software. The PCR and sequencing primers,

26 PCR amplification conditions, and sequence that was analysed, are listed in Supplementary

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Table S3. Any CpGs overlapping common SNPs were excluded from the analysis as they can represent a source of discrepancy.

All assay runs included standard DNA controls with 0%, 25%, 50%, 75% and 100% methylation status. Standards were prepared by diluting 100% methylated DNA (CpGenome Universal Methylated DNA, Millipore, UK) to 0% Whole Genome Amplified genomic DNA (Illustra GenomiPhi V2 DNA Amplification Kit, GE Healthcare Life Sciences). Whole genome amplified product was purified using the MinElute PCR Purification Kit (QIAGEN Ltd). 500ng of the mixed standards were bisulfite converted using both the EZ methylation and EZ-Lightning methylation kits (Zymo Research Corporation, Irvine, CA), according to the manufacturer's instructions. Each assay analysed two to six CpG sites. All the CpG sites within the assay, indicated the same directional effect on methylation levels.

Bisulfite treated DNA (1μl) was amplified in 25μl of PCR reaction mixture, containing 0.8μM of primers and 1U of Taq (FastStart DNA Polymerase, Roche). DNA was amplified in a heated-lid thermocycler as follows; 95°C for 5mins (x 1), 95°C for 30sec, annealing for 30sec at temperature corresponding to each set of primers, 72°C for 30sec (x 36), 72°C for 5mins (x 1). Single PCR products corresponding to the expected product size were confirmed by 2% agarose gel electrophoresis.

Sample preparation and pyrosequencing reactions were performed according to the manufacturer's instructions. Methylation values were quantified as percentage of methylated cytosine over the sum of methylated and unmethylated cytosines applying the formula (C/C + T) × 100, as determined by the Pyro Q-CpG™ software (QIAGEN).

Sample normality was checked using D'Agostino & Pearson omnibus K2 test; results with $p < 0.05$ were considered statistically significant and determined using unpaired, parametric t-test and one-way ANOVA analysis for samples with normal distribution and non-parametric Kruskal Wallis for skewed sample distributions. One-way ANOVA results were corrected for multiple testing using Bartlett's test, assuming a Gaussian distribution with similar standard

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1 deviations between populations (run automatically by Prism). Graph analysis is presented as

2 mean with 95% CI (confidence interval).

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4 *2.7. Data Annotation and Bioinformatics Analysis*

5 Annotations for the Infinium MethylationEPIC BeadChip were provided by Illumina

6 (https://support.illumina.com/array/array_kits/infinium-methylationepic-beadchip-

7 kit/downloads) and were based on GRCh37/hg19 build (Feb. 2009). Repeat Masker

8 (Institute for Systems Biology, repeatmasker.org) was used to exclude repeat elements

9 within primer sequences. Gene predictions were based on data from GENCODE v24

10 (currently hosted by the Ensembl Genome Browser), as well as RefSeq, Genbank, CCDS

11 and Uniprot (Known Genes dataset).

12 Information on the 106 differentially methylated CpG sites and their associated genes was

13 retrieved through mining the following databases; NCBI (www.ncbi.nlm.nih.gov), UCSC

14 Genome Browser (genome.ucsc.edu, both GRCh37/hg19 and GRCh38/hg38 builds),

15 meQTL database (http://www.mqtldb.org/) GeneCards suite (http://www.genecards.org/),

16 EnSEMBL (www.ensembl.org), EMBL-EBI (www.ebi.ac.uk), The ReproGenomics viewer

17 (rgv.genouest.org), Ovarian Kaleidoscope (okdb.appliedbioinfo.net), The Human Protein

18 Atlas (www.proteinatlas.org) and the Polycystic Ovary Syndrome database (pcosdb.net).

19 Gene predictions were based on data from GENCODE v24 (currently hosted by the Ensembl

20 Genome Browser), as well as RefSeq, Genbank, CCDS and Uniprot (Known Genes

21 dataset).

22

23 *2.8. Pathway and Gene Ontology Analyses*

24 Pathway and gene ontology analysis were carried out using the Ingenuity Pathway Analysis

25 (IPA) software (https://www.QIAGENbioinformatics.com/products/ingenuity-pathway-

1 analysis, (Kramer *et al.,* 2014)). A right-tailed Fisher's exact test was used to estimate

2 statistical significance ($p < 0.05$) for the network analysis.

3

4 **3. Results**

5 *3.1 EWAS approach and classification of differentially methylated CpG sites*

6 We performed a genome-wide, case-control epigenome profiling (EWAS), using the Illumina

7 Infinium MethylationEPIC BeadChip that covers > 850,000 methylation sites (Moran *et al.,*

8 2016) and DNA from GLCs, a known ovarian cell target for androgen action. Statistical

9 analysis of the EPIC array was performed using the CPACOR (incorporating Control Probe

10 Adjustment and reduction of global CORrelation) pipeline (Lehne *et al.*, 2015), with several

11 covariates.

12 Our EWAS sample population comprised a total of 32 participants (their clinical

13 characteristics are presented in Table S2). Age, smoking and body mass index (BMI) have

14 been known to affect global DNA methylation (Ashapkin *et al.,* 2017, Bell, 2017, Sundar *et*

15 *al.,* 2017). However, our study population comprised women of reproductive age, who were

16 predominantly non-smokers, and whose age and BMI were adjusted within a narrow range,

17 with around 4 years mean age difference between patients and controls, and very similar

18 normal BMI, in agreement with the eligibility criteria for receiving *In Vitro Fertilisation* (IVF)

19 treatment (National Institute for Health and Care Excellence guidelines (NICE)

20 https://www.nice.org.uk, last update-September 2017).

21 By using the presence or absence of PCOS ovarian morphology to compare datasets, we

22 identified 106 differentially methylated CpG sites between PCOS and controls ($p < 5.8 \times 10^{-8}$).

23 Correction of the analysis for one (CP1), or both (CP1 + CP2) internal control probes

24 identified 76 and 11 CpGs, respectively, that were largely overlapping with the 106 CpGs

25 originally identified (Supplementary Table S2). Since our study was based on a relatively

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1   small sample size (16 cases vs. 16 controls), we decided to consider all potential

2   differentially methylated CpG sites.

3   Using the follicular maturation trigger as an independent predictor of outcome, we identified

4   6 CpG sites with altered methylation. The type of trigger typically differs between women

5   with and without PCOS and so is a potential confounding variable, influenced by the PCOS

6   status. Women with PCOS typically require lower doses of FSH for ovarian stimulation; thus,

7   the cumulative dose of follicle-stimulating hormone (FSH) used during IVF treatment is

8   another possible confounder (our analysis identified two CpG sites that could be FSH dose-

9   dependent and therefore merit further investigation). Although the possible impact of age

10 and BMI was minimised by the selection criteria for IVF treatment, we also adjusted for

11 these, as well as for the total number of eggs collected, using all three covariates as

12 negative controls. In all cases we found no effect on methylation status.

13 Overall, amongst the 106 identified hits, the percentage of hyper- and hypo- methylated sites

14 was similar, with 52% of them appearing to be hypermethylated and 48% hypomethylated

15 (Supplementary Table S2). Apart from 18 CpGs that had intergenic locations (within either

16 repetitive elements, or regions with no annotated genes), the remaining 88 were localised in

17 the promoter regions and the body of validated and predicted genes, non-coding RNAs, and

18 some pseudogenes (Fig. 1 and 2).

19 Database search indicated that these 88 genes were predominantly involved in gene

20 regulation, endocrine and metabolic functions, immune response, cell signalling, cell death

21 and survival and structural integrity (Supplementary Table S2). Of those, 36 genes are part

22 of pathways relevant to PCOS phenotype, such as metabolic, cardiovascular, circadian,

23 neurological and endocrine systems, or have an ovary related function (Fig. 3,

24 Supplementary Table S2). Although there is a certain degree of complexity regarding the

25 effect of methylation status on gene expression (Khalaf *et al.,* 2013, Louwers *et al.,* 2013,

26 Yang *et al.,* 2014), the identification of differential methylation in genes like *BMPR1A,*

11

FERMT2 and *HMGA2* that have been directly associated with the PCOS phenotype is very encouraging.

Of the106 identified CpGs, results from 6 were validated using pyrosequencing, with the addition of extra cases and control samples with similar clinical characteristics as those included in the microarray experiment (see Materials and Methods, Tables 1 and 2). These targeted selections were based on the degree of statistical significance (all target p-values were ≤ $1.66e^{-08}$), genomic location, relationship with ovarian functions and PCOS involvement.

Pyrosequencing was also used to assess the methylation of a PCOS sub-group, comprising patients with regular cycles and compare the pattern with PCOS patients with oligomenorrhea, as well as healthy controls. There were no significant differences between the two PCOS groups, indicating that the two sub-phenotypes appear, at least for the examined CpGs, epigenetically similar (Fig. 4).

3.2 Pathway and network analysis

To assess the biological relevance and potential functional interactions of our findings, we carried out a core pathway analysis, using the Ingenuity Pathway Analysis software (IPA). A comparative review indicated 20 potentially disrupted canonical pathways with p-values ranging from 5.9×10^{-4} to 5.0×10^{-2}. The most statistically significant pathways that were identified included molecular mechanisms of cancer, cardiogenesis, Sonic Hedgehog signalling, immune response and mitochondrial dysfunction, with some genes being present in more than one pathway (Fig. 5).

4. Discussion

PCOS is a polygenic disorder with a complex mode of inheritance. To date, the PCOS phenotype has been associated with 241 genes ((Joseph *et al.,* 2016),

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1   http://pcoskb.bicnirrh.res.in) and 16 PCOS susceptibility loci, encompassing genes involved

2   in neuroendocrine, metabolic and reproductive functions (Brower *et al.,* 2015, Chen *et al.*,

3   2011, Day *et al.*, 2015, Day *et al.*, 2018, Hayes *et al.*, 2015, Li *et al.,* 2012, Louwers *et al.*,

4   2013, Shi *et al.*, 2012). Nevertheless, the complexity and heterogeneity by which the disease

5   appears amongst the female population remains elusive.

6   To our knowledge, this PCOS epigenome wide association (EWAS) study is the first

7   documented that uses GLCs, an ovary specific cell type, in combination with Illumina's EPIC

8   array that screens >850,000 methylation sites, doubling the number from their previous

9   version of 450K. A similar study from Xu *et al.,* 2016, also utilised GLCs for their analysis but

10 on the 450K array platform and without adjusting for multiple comparisons. In contrast, our

11 study is based on data that have been thoroughly interrogated, corrected for genome-wide

12 multiple comparisons and adjusted through the application of several normalisation methods

13 and linear prediction models. Nevertheless, since both studies were performed using GLCs

14 and overlapping Illumina arrays, we were able to directly compare the Xu *et al.* findings with

15 our data (Supplementary Tables S3 and S4), identifying several CpGs in our study that were

16 replicated in all three Xu *et al.* group analyses, as follows; 1) Controls versus PCOS obesity;

17 108/1472 replicated (p-value = 6.6 x 10^{-5}), 2) Controls versus PCOS non-obesity; 155/2471

18 replicated (p-value = 0.003), 3) PCOS non-obesity vs. PCOS obesity; 45/1089 replicated (p-

19 value= 0.9).

20 Overall, we identified 106 CpGs with differential methylation between PCOS patients and

21 healthy controls. Of these, 88 were associated with genes, several of which are implicated in

22 endocrine, metabolic and reproductive processes found to be associated with PCOS. In

23 addition, 16 of the identified CpGs were mapped within 6 known PCOS susceptibility loci and

24 of those, 5 were overlapping with known methylation quantitative trait loci (meQTLs) (Fig. 6,

25 Supplementary Table S2) that may affect gene expression levels in a cis- or trans- acting

26 fashion, strengthening the notion of a plausible environmental contribution to the genetic

27 basis of PCOS (Schalkwyk *et al.,* 2010, Shoemaker *et al.,* 2010).

1   We validated our results using pyrosequencing targeted analysis of 6 specific CpG sites

2   from the same patient-control cohort used in the genome wide approach, with the addition of

3   extra samples from both patients and controls (see Methods and Materials, Table 1). Overall,

4   the results from both experimental platforms were in agreement, having the same direction

5   of effect, but with lower significance levels for the pyrosequencing experiment (Fig. 4). A

6   possible explanation would be the use of two different experimental platforms and multiple

7   batches of bisulfite treated DNA for each sample, both of which can add layers of technical

8   bias and introduce a degree of variability in the replication of results.

9   In addition, using pyrosequencing, the CpG site associated with FLJ4034, the selected

10 target with the most significant p-value, failed to replicate. FLJ4034 is a potential

11 pseudogene and determination of its methylation status can be ambiguous due to the high

12 methylation levels that pseudogenes usually exhibit in order to be silenced. Indeed, our

13 analysis indicated >95% methylation for FLJ4034 in both cases and controls.

14 A prominent feature of PCOS is its clinical heterogeneity that can easily result in a complex

15 or inaccurate diagnosis. The main reason for such heterogeneity is that PCOS development

16 and progression are controlled by several mechanisms and environmental factors, involving

17 genes expressed at various degrees, in a multitude of ovarian cell types. Significantly, we

18 found the methylation profile of PCOS patients with and without menstrual irregularities to be

19 similar, suggesting a shared epigenetic and potentially genetic architecture between distinct

20 sub-phenotypes (Fig. 4). This mirrors findings in a recent meta-analysis of GWAS studies in

21 which there was lack of genetic heterogeneity between women with PCOS irrespective of

22 the diagnostic criteria that were applied (Day *et al.*, 2018).

23 Pathway analysis identified CpG-associated genes from our panel, as part of potential

24 molecular synergies that could shed some light to the disease heterogeneity (Fig. 5, Table

25 3).

14

The top five Identified signalling pathways include cardiogenesis, Sonic Hedgehog, Nur77, mitochondrial dysfunction and cancer, with pathologies like endometrial cancer (shown to be more common in women with PCOS), cardiovascular disease, oocyte growth and maturation, and immune response, all of which have been associated with the development of PCOS. It is not surprising that "Molecular mechanisms to cancer" was identified as the most important pathway, with the involvement of seven genes from our dataset. It is well known that epigenetic alterations can affect gene expression and create genetic instability, disrupting signalling cascades and predisposing not only to malignant phenotypes, but also to a wide spectrum of pathologies. Of significance is also the fact that a potential involvement of immune response to the PCOS development is - further to pathway analysis - supported by data mining of our dataset, where we identified a total of 16 CpG-associated genes involved in immune and inflammatory response (Supplementary Table S2). Given that the GLC purification procedure has removed any carry-over cells, including lymphocytes that could bias the results, our findings suggest that immune response may play an important role in the diversification of PCOS.

5. Conclusion

Over the past decade our view has shifted from the dogma of "genetic determinism" to a more flexible and interactive scenario of constant and dynamic relationship of our genome with the environment. It is therefore becoming increasingly evident that epigenetic modification is a crucial component of our genetic make-up and plays a critical role in key regulatory processes, genome stability and organismal adaptation to environmental exposure (Bjornsson, 2015).

These 106 differentially methylated CpGs present the opportunity, through further investigation, to be developed into diagnostic, as well as prognostic landmarks and risk indicators, since epigenetic changes can precede disease manifestation. Most importantly,

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epigenetic changes are environmentally dependent; hence, altering environmental exposure

can reverse the effect, making them candidates for therapeutic agents (Cole *et al.,* 2017,

Kanwal *et al.,* 2015, Nicoll *et al.,* 2001, Teruel and Sawalha, 2017).

Supplementary data access

The raw. IDAT files from the genome-wide methylation study using the EPIC Beadchip array,

have been deposited to ArrayExpress Data Warehouse

(https://www.ebi.ac.uk/arrayexpress/), with accession number: E-MTAB-6672.

References

Abbott DH, Barnett DK, Bruns CM, Dumesic DA. Androgen excess fetal programming of female
reproduction: a developmental aetiology for polycystic ovary syndrome? *Hum Reprod Update*
2005;**11**:357-374.
Abbott DH, Dumesic DA, Franks S. Developmental origin of polycystic ovary syndrome - a hypothesis.
J Endocrinol 2002;**174**:1-5.

16

Adams J, Liu Z, Ren YA, Wun WS, Zhou W, Kenigsberg S, Librach C, Valdes C, Gibbons W, Richards J. Enhanced Inflammatory Transcriptome in the Granulosa Cells of Women With Polycystic Ovarian Syndrome. *J Clin Endocrinol Metab* 2016;**101**:3459-3468.

Ashapkin VV, Kutueva LI, Vanyushin BF. Aging as an Epigenetic Phenomenon. *Curr Genomics* 2017;**18**:385-407.

Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. *J Clin Endocrinol Metab* 2004;**89**:2745-2749.

Barber TM, Franks S. Genetics of polycystic ovary syndrome. *Front Horm Res* 2013;**40**:28-39.

Bell CG. The Epigenomic Analysis of Human Obesity. *Obesity (Silver Spring)* 2017;**25**:1471-1481.

Bjornsson HT. The Mendelian disorders of the epigenetic machinery. *Genome Res* 2015;**25**:1473-1481.

Brower MA, Jones MR, Rotter JI, Krauss RM, Legro RS, Azziz R, Goodarzi MO. Further investigation in europeans of susceptibility variants for polycystic ovary syndrome discovered in genome-wide association studies of Chinese individuals. *J Clin Endocrinol Metab* 2015;**100**:E182-186.

Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J *et al.* Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet* 2011;**43**:55-59.

Cole E, Brown TA, Pinkerton KE, Postma B, Malany K, Yang M, Kim YJ, Hamilton RF, Jr., Holian A, Cho YH. Perinatal exposure to environmental tobacco smoke is associated with changes in DNA methylation that precede the adult onset of lung disease in a mouse model. *Inhal Toxicol* 2017;**29**:435-442.

Coskun S, Otu HH, Awartani KA, Al-Alwan LA, Al-Hassan S, Al-Mayman H, Kaya N, Inan MS. Gene expression profiling of granulosa cells from PCOS patients following varying doses of human chorionic gonadotropin. *J Assist Reprod Genet* 2013;**30**:341-352.

Day F Karaderi T, Jones M, Meun C, He C, Drong A, Kraft P, Lin N, Huang H, Broer L *et al.* Large-Scale Genome-Wide Meta Analysis of Polycystic Ovary Syndrome Suggests Shared Genetic Architecture for Different Diagnostic Criteria. *PLOS Genetics* 2018;**In press**.

Day FR, Hinds DA, Tung JY, Stolk L, Styrkarsdottir U, Saxena R, Bjonnes A, Broer L, Dunger DB, Halldorsson BV *et al.* Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome. *Nat Commun* 2015;**6**:8464.

Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. *Endocr Rev* 2012;**33**:981-1030.

Fortin JP, Triche TJ, Jr., Hansen KD. Preprocessing, normalization and integration of the Illumina HumanMethylationEPIC array with minfi. *Bioinformatics* 2017;**33**:558-560.

Franks S. Polycystic ovary syndrome. *N Engl J Med* 1995;**333**:853-861.

Franks S, Berga SL. Does PCOS have developmental origins? *Fertil Steril* 2012;**97**:2-6.

Franks S, Gharani N, Waterworth D, Batty S, White D, Williamson R, McCarthy M. The genetic basis of polycystic ovary syndrome. *Hum Reprod* 1997;**12**:2641-2648.

Franks S, Webber LJ, Goh M, Valentine A, White DM, Conway GS, Wiltshire S, McCarthy MI. Ovarian morphology is a marker of heriTable biochemical traits in sisters with polycystic ovaries. *J Clin Endocrinol Metab* 2008;**93**:3396-3402.

Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, Karaderi T, Barber TM, McCarthy MI, Franks S *et al.* Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. *Nat Commun* 2015;**6**:7502.

Insenser M, Escobar-Morreale HF. Application of proteomics to the study of polycystic ovary syndrome. *J Endocrinol Invest* 2011;**34**:869-875.

Jayasena CN, Franks S. The management of patients with polycystic ovary syndrome. *Nat Rev Endocrinol* 2014;**10**:624-636.

Joseph S, Barai RS, Bhujbalrao R, Idicula-Thomas S. PCOSKB: A KnowledgeBase on genes, diseases, ontology terms and biochemical pathways associated with PolyCystic Ovary Syndrome. *Nucleic Acids Res* 2016;**44**:D1032-1035.

17

1   Kanwal R, Gupta K, Gupta S. Cancer epigenetics: an introduction. *Methods Mol Biol* 2015;**1238**:3-25.

2   Khalaf M, Morera J, Bourret A, Reznik Y, Denoual C, Herlicoviez M, Mittre H, Benhaim A. BMP system

3   expression in GCs from polycystic ovary syndrome women and the in vitro effects of BMP4, BMP6,

4   and BMP7 on GC steroidogenesis. *Eur J Endocrinol* 2013;**168**:437-444.

5   Kokosar M, Benrick A, Perfilyev A, Fornes R, Nilsson E, Maliqueo M, Behre CJ, Sazonova A, Ohlsson C,

6   Ling C *et al.* Epigenetic and Transcriptional Alterations in Human Adipose Tissue of Polycystic Ovary

7   Syndrome. *Sci Rep* 2016;**6**:22883.

8   Kramer A, Green J, Pollard J, Jr., Tugendreich S. Causal analysis approaches in Ingenuity Pathway

9   Analysis. *Bioinformatics* 2014;**30**:523-530.

10   Lehne B, Drong AW, Loh M, Zhang W, Scott WR, Tan ST, Afzal U, Scott J, Jarvelin MR, Elliott P *et al.* A

11   coherent approach for analysis of the Illumina HumanMethylation450 BeadChip improves data

12   quality and performance in epigenome-wide association studies. *Genome Biol* 2015;**16**:37.

13   Li L, Zhang J, Deng Q, Li J, Li Z, Xiao Y, Hu S, Li T, Tan Q, Li X *et al.* Proteomic Profiling for Identification

14   of Novel Biomarkers Differentially Expressed in Human Ovaries from Polycystic Ovary Syndrome

15   Patients. *PLoS One* 2016;**11**:e0164538.

16   Li S, Zhu D, Duan H, Ren A, Glintborg D, Andersen M, Skov V, Thomassen M, Kruse T, Tan Q.

17   Differential DNA methylation patterns of polycystic ovarian syndrome in whole blood of Chinese

18   women. *Oncotarget* 2017;**8**:20656-20666.

19   Li T, Zhao H, Zhao X, Zhang B, Cui L, Shi Y, Li G, Wang P, Chen ZJ. Identification of YAP1 as a novel

20   susceptibility gene for polycystic ovary syndrome. *J Med Genet* 2012;**49**:254-257.

21   Li Z, Huang H. Epigenetic abnormality: a possible mechanism underlying the fetal origin of polycystic

22   ovary syndrome. *Med Hypotheses* 2008;**70**:638-642.

23   Louwers YV, Stolk L, Uitterlinden AG, Laven JS. Cross-ethnic meta-analysis of genetic variants for

24   polycystic ovary syndrome. *J Clin Endocrinol Metab* 2013;**98**:E2006-2012.

25   Lv Y, Zhao SG, Lu G, Leung CK, Xiong ZQ, Su XW, Ma JL, Chan WY, Liu HB. Identification of reference

26   genes for qRT-PCR in granulosa cells of healthy women and polycystic ovarian syndrome patients. *Sci*

27   *Rep* 2017;**7**:6961.

28   Mani H, Levy MJ, Davies MJ, Morris DH, Gray LJ, Bankart J, Blackledge H, Khunti K, Howlett TA.

29   Diabetes and cardiovascular events in women with polycystic ovary syndrome: a 20-year

30   retrospective cohort study. *Clin Endocrinol (Oxf)* 2013;**78**:926-934.

31   March WA, Moore VM, Willson KJ, Phillips DI, Norman RJ, Davies MJ. The prevalence of polycystic

32   ovary syndrome in a community sample assessed under contrasting diagnostic criteria. *Hum Reprod*

33   2010;**25**:544-551.

34   Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of

35   the human genome enriched in enhancer sequences. *Epigenomics* 2016;**8**:389-399.

36   Nicoll G, Crichton DN, McDowell HE, Kernohan N, Hupp TR, Thompson AM. Expression of the

37   Hypermethylated in Cancer gene (HIC-1) is associated with good outcome in human breast cancer.

38   *Br J Cancer* 2001;**85**:1878-1882.

39   Qu F, Wang FF, Yin R, Ding GL, El-Prince M, Gao Q, Shi BW, Pan HH, Huang YT, Jin M *et al.* A

40   molecular mechanism underlying ovarian dysfunction of polycystic ovary syndrome:

41   hyperandrogenism induces epigenetic alterations in the granulosa cells. *J Mol Med (Berl)*

42   2012;**90**:911-923.

43   Rotterdam EA-SPCWG. Revised 2003 consensus on diagnostic criteria and long-term health risks

44   related to polycystic ovary syndrome. *Fertil Steril* 2004;**81**:19-25.

45   Schalkwyk LC, Meaburn EL, Smith R, Dempster EL, Jeffries AR, Davies MN, Plomin R, Mill J. Allelic

46   skewing of DNA methylation is widespread across the genome. *Am J Hum Genet* 2010;**86**:196-212.

47   Shen HR, Qiu LH, Zhang ZQ, Qin YY, Cao C, Di W. Genome-wide methylated DNA

48   immunoprecipitation analysis of patients with polycystic ovary syndrome. *PLoS One* 2013;**8**:e64801.

49   Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, Zhang B, Liang X, Li T, Chen J *et al.* Genome-wide association

50   study identifies eight new risk loci for polycystic ovary syndrome. *Nat Genet* 2012;**44**:1020-1025.

1   Shoemaker R, Deng J, Wang W, Zhang K. Allele-specific methylation is prevalent and is contributed
2   by CpG-SNPs in the human genome. *Genome Res* 2010;**20**:883-889.
3   Sirmans SM, Pate KA. Epidemiology, diagnosis, and management of polycystic ovary syndrome. *Clin*
4   *Epidemiol* 2013;**6**:1-13.
5   Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential
6   expression in microarray experiments. *Bioinformatics* 2005;**21**:2067-2075.
7   Sundar IK, Yin Q, Baier BS, Yan L, Mazur W, Li D, Susiarjo M, Rahman I. DNA methylation profiling in
8   peripheral lung tissues of smokers and patients with COPD. *Clin Epigenetics* 2017;**9**:38.
9   Teruel M, Sawalha AH. Epigenetic Variability in Systemic Lupus Erythematosus: What We Learned
10 from Genome-Wide DNA Methylation Studies. *Curr Rheumatol Rep* 2017;**19**:32.
11 Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI. Heritability of polycystic ovary syndrome in a Dutch
12 twin-family study. *J Clin Endocrinol Metab* 2006;**91**:2100-2104.
13 Wang XX, Wei JZ, Jiao J, Jiang SY, Yu DH, Li D. Genome-wide DNA methylation and gene expression
14 patterns provide insight into polycystic ovary syndrome development. *Oncotarget* 2014;**5**:6603-
15 6610.
16 Xita N, Tsatsoulis A. Review: fetal programming of polycystic ovary syndrome by androgen excess:
17 evidence from experimental, clinical, and genetic association studies. *J Clin Endocrinol Metab*
18 2006;**91**:1660-1666.
19 Xu J, Bao X, Peng Z, Wang L, Du L, Niu W, Sun Y. Comprehensive analysis of genome-wide DNA
20 methylation across human polycystic ovary syndrome ovary granulosa cell. *Oncotarget*
21 2016;**7**:27899-27909.
22 Xu N, Azziz R, Goodarzi MO. Epigenetics in polycystic ovary syndrome: a pilot study of global DNA
23 methylation. *Fertil Steril* 2010;**94**:781-783 e781.
24 Yu YY, Sun CX, Liu YK, Li Y, Wang L, Zhang W. Genome-wide screen of ovary-specific DNA methylation
25 in polycystic ovary syndrome. *Fertil Steril* 2015;**104**:145-153 e146.

26 Yang M, Du J, Lu D, Ren C, Shen H, Qiao J, Chen X, Zhang H. Increased expression of kindlin 2 in
27 luteinized granulosa cells correlates with androgen receptor level in patients with polycystic ovary
28 syndrome having hyperandrogenemia. *Reprod Sci* 2014;**21**:696-703

29

30 **Figures and figure captions**
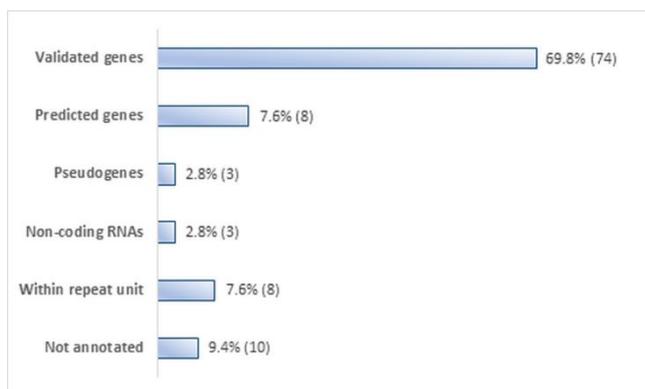
31

32 **Fig. 1.** Localisation of the 106 identified CpGs within the genome and their association

33 (numbers and percentages) with types of genes (validated, predicted, pseudogenes, non-

34 coding RNAs), repeat units and random non-annotated genomic locations.

35
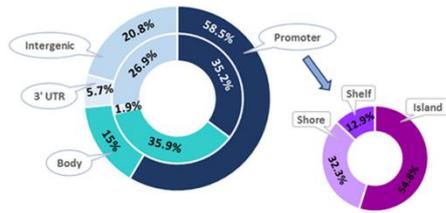
1

2 **Fig. 2.** Distribution within the genome of the 106 CpG sites identified in our study (outside

3 ring) and the published EPIC design coverage (Moran *et al.*, 2016) (inside ring). Genomic

4 locations are categorised as gene related (promoter, body, 3'UTR) and intergenic (no

5 annotated genes in close vicinity). Promoter related sites are further subcategorised

6 depending on the CpG site location (island, shore, self).

7
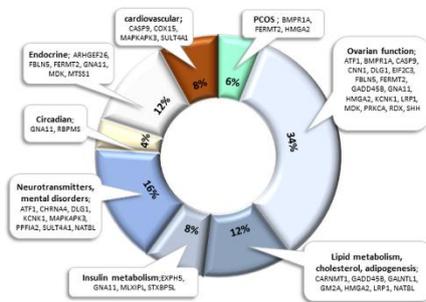
8

9 **Fig. 3.** Graphical representation of the 36 gene-associated CpG sites identified in our

10 analysis that are directly, or indirectly related to PCOS. Some genes are present in more

11 than one category; hence percentage calculations are based on a total of 50 entries.

12

20
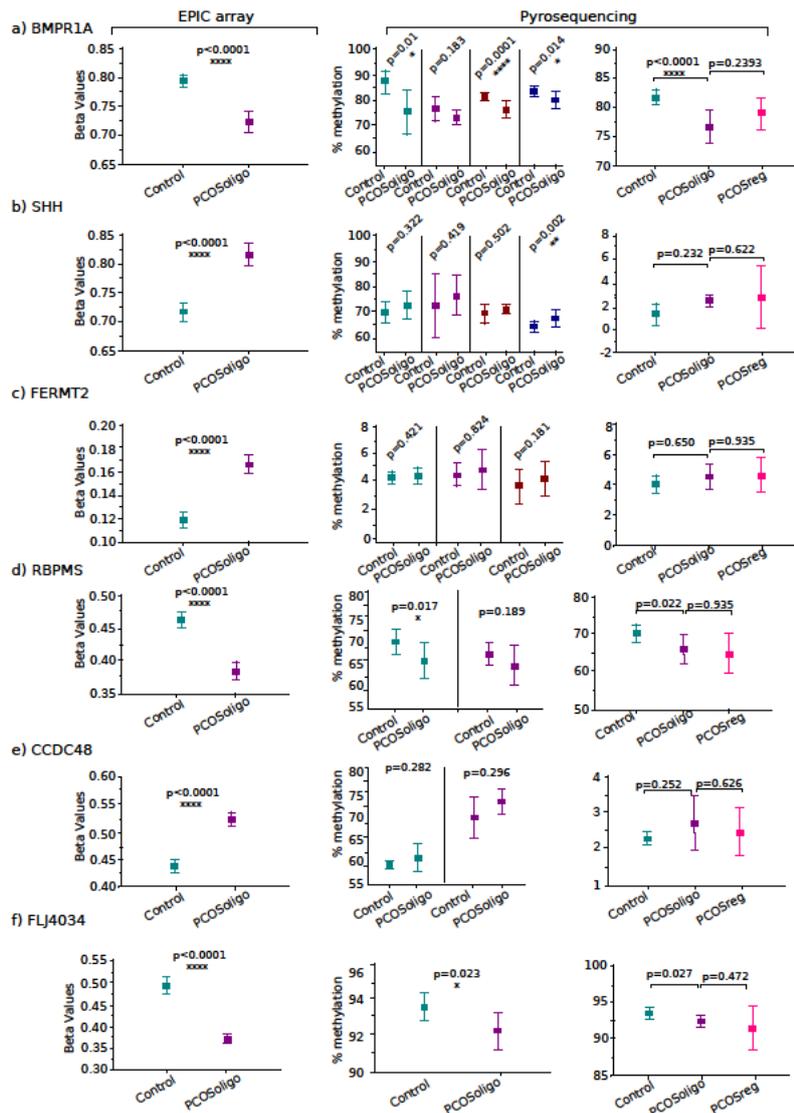
Fig. 4. Box plot graphs of differential methylation levels of 6 differentially methylated CpG sites between two sub-groups of PCOS patients and healthy controls. Left hand graphs: microarray analysis adjusting for PCOS ovarian morphology; Y-axis: beta values. Middle graphs: pyrosequencing analysis using DNA from PCOS patients with oligomenorrhea; Y-axis: % methylation levels. Right hand graphs: pyrosequencing analysis using DNA from patients with regular cycles; Y-axis: % methylation levels. Expts 1, 2, 3, 4; number of pyrosequencing assays repeated for the same CpG methylation target.

21
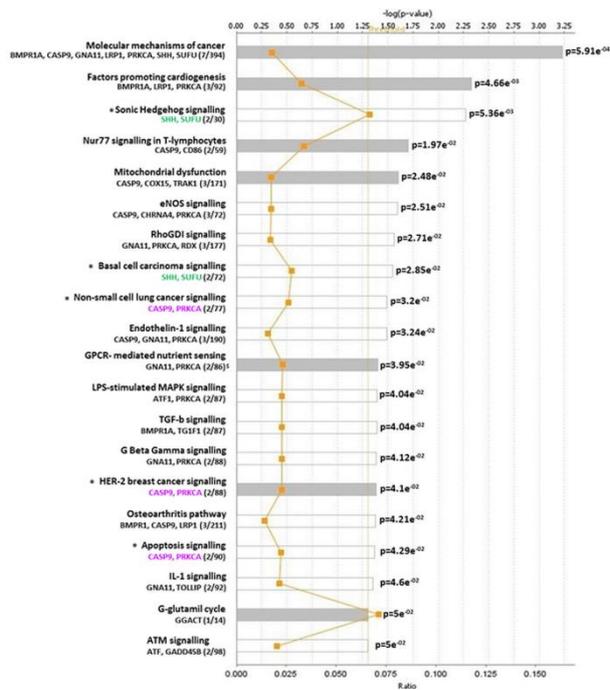
Fig. 5. Pathway analysis of the 88 gene-associated differentially methylated CpGs. Y-axis; significant canonical pathways for the dataset. X-axis; -log (p value), calculated by Fisher's right-tailed exact test. The ratio (orange line) is calculated as number of genes in a given pathway that meet cut-off criteria, divided by the total number of genes that make up that pathway. Threshold indicates the fraction of false positives among significant functions. Pathways that have a –log (p value) greater than the threshold of 1.3 (range 0 to 3.23) are displayed to the right-hand side of the graph. White bars; z-score at, or close to 0. Grey bars; pathways with no current prediction.

22

Locus	Reference	Gene	Ancestry	Epic Hits	Associated Genes	Relative Gene Distance
11q.22.1	Day et al. 2015 Chen ZJ et al. 2011 Li et al. 2012	YAP1	European Han Chinese	2	EXPH5 (**mQTL**), RDX	
12q.13.2	Day et al. 2015 Chen ZJ et al. 2011	RAB5B / SUOX	European Han Chinese	3	ATF1, LRP1 (**mQTL**), NCKAP5L	
12q.14.3	Day et al. 2015 Chen ZJ et al. 2011 Hayes et al. 2015 Louwers et al 2013	HMGA2	European Han Chinese	1	HMGA2	
12q.21.2	Hayes et al. 2015	KRR1	European	1	PPFIA2	
19p.13.3	Chen ZJ et al. 2011 Brower et al. 2015	INSR	European Han Chinese	7	CELF5 (**mQTL**), GADD45B, GNA11, CNN1 (**mQTL**), MUM1 (x2), SLC25A23 (**mQTL**)	
20q.13.2	Chen ZJ et al. 2011	SUMO1P1	Han Chinese	2	CHRNA4, RP5-1030M6.3	

Fig. 6. Mapping of 16 gene-associated CpGs within published PCOS susceptibility loci.
Table lists information regarding the susceptibility loci (chromosomal region, publication,
published gene/s and ancestry used for the GWAS study) and the number of CpGs from the
analysis located within these regions, the name of the associated to the CpGs genes and a
graphical representation of their localisation within the genome.

23

1 **Table 1** Clinical characteristics of PCOS patients and controls used for the array-based and the pyrosequencing analysis. The pyrosequencing
2 analysis includes all samples used for the genome-wide study, extra samples for each group and an extra group comprising women suffering
3 from PCOS, but with regular cycles. Group comparisons were performed using a two-tailed, unpaired t-test. Values are mean, ± standard error
4 of means (SEM). N: number of patients, GnRHA: Gonadotropin-releasing hormone agonist, hCG: Human chorionic gonadotrophin, BMI: Body
5 Mass Index, LH: Luteinizing Hormone, FSH: Follicle-Stimulating Hormone, Hep B, C: Hepatitis B, C, HIV: Human Immunodeficiency Virus.
6 Number of * indicate level of significance

	EPIC array cohort			Pyrosequencing cohort			
	PCOS	*Controls*	*P-value*	*PCOS*	*Controls*	*PCOSreg*	*P-value*
Menstrual cycle	Irregular	Regular		Irregular	Regular		
Ovarian morphology	PCO	Normal		PCO	Normal	PCO	
Mean age (y)	30.19 ± 0.8, N=16	34.75 ± 0.9, N=16	0.0009***	30.2 ± 0.6, N=26	35.1 ± 0.6, N=43	32.9 ± 1.1, N=10	<0.0001****
Mean BMI (kg/m2)	24.89 ± 0.8, N=16	24.38 ± 0.5, N=16	0.593	24.5 ± 0.6, N=26	24.4 ± 0.4, N=43	23.6 ± 1.1, N=10	0.714
Protocol	Antagonist	Long agonist, Antagonist		Antagonist	Long agonist, Antagonist	Antagonist	
Gonadotropin used	FSH (Gonal-f)	FSH (Gonal-f)		FSH (Gonal-f)	FSH (Gonal-f)	FSH (Gonal-f)	
Maturation trigger	GnRHA, Kisspeptin	hCG		GnRHA, Kisspeptin	hCG	GnRHA, Kisspeptin	
No. of antral follicles	36 ± 3.4, N=16	14 ± 1.4, N=16	<0.0001****	38.6 ± 2.9, N=26	12.2 ± 0.9, N=43	28.4 ± 2.1, N=10	<0.0001****
Baseline FSH (mIU/mL)	5.2 ± 0.8, N=15	6.3 ± 0.6, N=12	0.2645	5.3 ± 0.5, N=24	6.5 ± 0.5, N=30	4.4 ± 0.7, N=9	0.0555
Baseline LH (mIU/mL)	5.3 ± 0.9, N=15	4.3 ± 0.4, N=10	0.4101	6.8 ± 0.9, N=23	5 ± 0.43 N=28	5.1 ± 0.9, N=9	0.1114
Days of stimulation	11.4 ± 0.8, N=16	11.1 ± 0.5, N=16	0.7815	11.1 ± 0.5, N=26	11.1 ± 0.3, N=43	11.3 ± 1.3, N=10	0.9674
Cumulative FSH dose (iu)	1578 ± 198, N=16	2986 ± 296, N=16	0.0004***	1466.3 ± 132, N=26	3087.2 ± 183, N=43	1551.5 ± 121.4, N=10	<0.0001****
No. of follicles >14mm	15 ± 1.2, N=16	8 ± 0.7, N=16	<0.0001****	15 ± 1.5, N=26	7.6 ± 0.6, N=43	9.8 ± 1.9, N=10	<0.0001****
No. of eggs collected	20 ± 3.3, N=16	12 ± 1.3, N=16	0.0263*	18.8 ± 2.3, N=26	10 ± 0.8, N=43	15.2 ± 2.8, N=10	0.0002***
Smokers	0	2		0	2	0	
Alcohol drinking	1	4		2	6	0	
Hep B, C, HIV +ve	1	0		1	0	0	
Family history	No	No		No	No	No	
Medical history	No	Migraines (1), asthma (1), epilepsy (1), hypothyroidism (1), Raynaud's syndrome (1)		No	Migraines (2), asthma (1), epilepsy (1), hypothyroidism (1), Raynaud's syndrome (1)	No	

24

Table 2 Statistical analysis results and associated gene details on 10 differentially methylated CpG sites chosen for pyrosequencing validation. $^{\$}$ denotes closest gene to the CpG of interest, * N_ and S_ denote the upstream and downstream end of the island region. P-value corresponds to linear regression analysis of the EPIC array.

Illumina ID	$^{\$}$Gene	chr	p-value	Comments	Disease	Ovary	PCOS	*CpG location
cg11683966	FLJ40434	1p.32	2.09e^{-14}	Pseudogene				S_Shore
cg00112465	CCDC48	3q.21	7.04e^{-13}	Predicted gene				Island
cg18364576	FERMT2	14q.22	3.44e^{-10}	Scaffold protein related to PCOS androgen receptors	Hyper-androgenemia	Yes	Yes	N_Shore
cg10821050	SHH	7q36	1.40e^{-09}	Follicle and early embryo development, signal mediator btw granulosa and theca cells	Cancer, developmental disorders	Yes		N_Shore
cg23044884	RBPMS	8p.12	1.39e^{-08}	Transcriptional regulator				S_Shelf
cg12976821	BMPR1A	10q22	1.66e^{-08}	TGF-b pathway downregulated by testosterone	Polyposis, Thyroid	Yes	Yes	5'UTR

25

Table 3 List of the top five Identified signalling pathways with CpG-associated genes from our EWAS analysis

	IPA Pathway	*Genes from our dataset*	*PCOS associated pathology or gene*	*References*
# 1	Molecular mechanisms of cancer	*BMPR1A*, *CASP9*, *GNA11*, *LRP1*, *PRKCA*, *SHH* and *SUFU*	A well-recognised PCOS associated pathology is endometrial cancer	Shen et al. 2013
# 2	Cardiogenesis	*BMPR1A*, *LRP1* and *PRKCA*	BMPR1A is PCOS related and LRP1 is involved in lipid metabolism. Also, PCOS is frequently accompanied by an increased risk for cardiovascular disease, because of molecular interactions between obesity, testosterone and dyslipidemia	*Couto Alves et al. 2017*
# 3	Sonic Hedgehog (SH) signalling	*SHH* and *SUFU*	SH is involved in ovarian follicular growth, regulating the steroidogenic capacity of endocrine cells like GLCs and signalling oocyte maturation, especially towards the final stages of antral follicle development. One of the key features of PCOS is the production of multiple prematurely arrested follicles leading to reduced fertility. Therefore, it is plausible that SH may be implicated in the development of anovulation in PCOS	Wijgerde et al. 2005 Spicer et al. 2009 Hatzirodos et al. 2014 Wang et al. 2017
# 4	Nur77, T-lymphocytes signalling	*CASP9* and *CD86*	Nur77 is related to autoimmune response prevention. PCOS development and progression have been associated with immune response. It has been hypothesized that functional autoantibodies lead to a higher prevalence of autoimmune thyroiditis amongst PCOS patients.	Gleicher et al. 2007 Arora et al. 2016 Ferreira and Motta 2017 Tremellen et al. 2017 Xu et al. 2017

			Also, several studies have indicated a link between increased androgen levels in PCOS patients, and inflammatory conditions, both systemic and GLC localised	
#5	Mitochondrial dysfunction	*CASP9, COX15* and *TRAK1*	Mitochondrial biogenesis is particularly important during oocyte growth and its impairment has been associated with poor oocyte quality, diminished ovarian reserves and insulin resistance. Although patients selected for the study did not suffer from insulin resistance or hyperinsulinemia, these findings suggest a potential predisposition to insulin signalling disruption that may prove functionally significant given that methylation changes can precede clinical manifestation of the disease by several years	Wang et al. 2010 Wang and Moley 2010 Boucret et al. 2015 Zhao et al. 2015 Ding et al. 2017 Mimura et al. 2016 Cai et al. 2018

27

Table 1

Table 1 Clinical characteristics of PCOS patients and controls used for the array-b

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Alcohol drinking	1	4	
Hep B, C, HIV +ve	1	0	
Family history	No	No	
Medical history	No	Migraines (1), asthma (1), epilepsy (1), hypothyroidism (1), Raynaud's syndrome (1)	

Table 2

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Figure 1
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Validated genes — 69.8% (74)
Predicted genes — 7.6% (8)
Pseudogenes — 2.8% (3)
Non-coding RNAs — 2.8% (3)
Within repeat unit — 7.6% (8)
Not annotated — 9.4% (10)
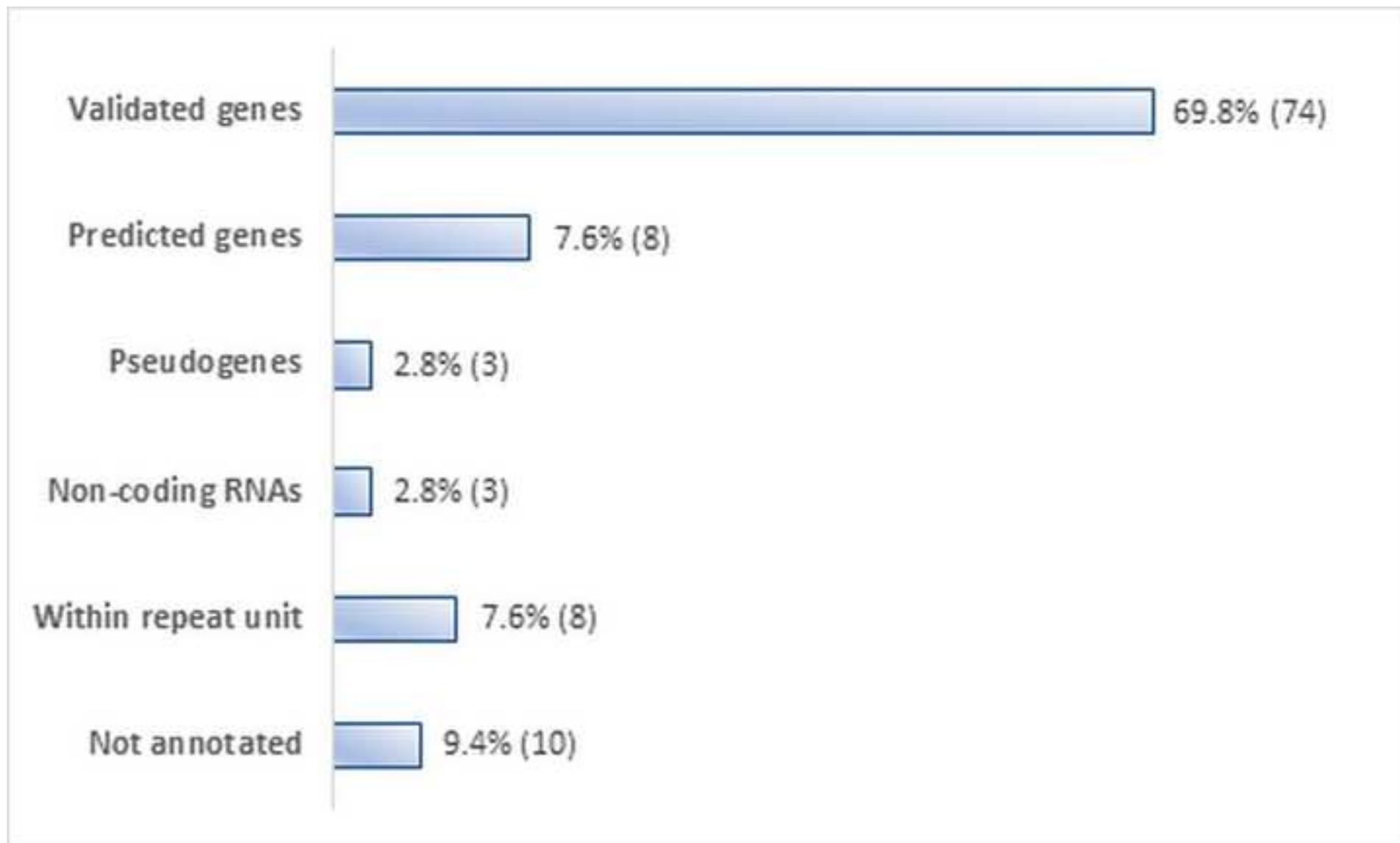
Figure 2
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Figure 3

cardiovascular;
CASP9, COX15,
MAPKAPK3, SULT4A1

PCOS ; BMPR1A,
FERMT2, HMGA2

Endocrine; ARHGEF26,
FBLN5, FERMT2, GNA11,
MDK, MTSS1

Ovarian function;
ATF1, BMPR1A, CASP9,
CNN1, DLG1, EIF2C3,
FBLN5, FERMT2,
GADD45B, GNA11,
HMGA2, KCNK1, LRP1,
MDK, PRKCA, RDX, SHH

Circadian;
GNA11, RBPMS

Neurotransmitters,
mental disorders;
ATF1, CHRNA4, DLG1,
KCNK1, MAPKAPK3,
PPFIA2, SULT4A1, NAT8L

Lipid metabolism,
cholesterol, adipogenesis;
CARNMT1, GADD45B, GALNTL1,
GM2A, HMGA2, LRP1, NAT8L

Insulin metabolism;EXPH5,
GNA11, MLXIPL, STXBP5L

8% 6% 12% 4% 34% 16% 8% 12%

Figure 4

a) BMPR1A
b) SHH
c) FERMT2
d) RBPMS
e) CCDC48
f) FLJ4034
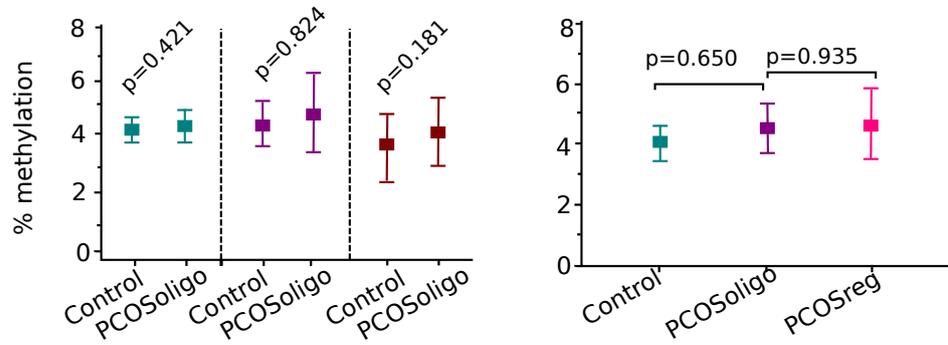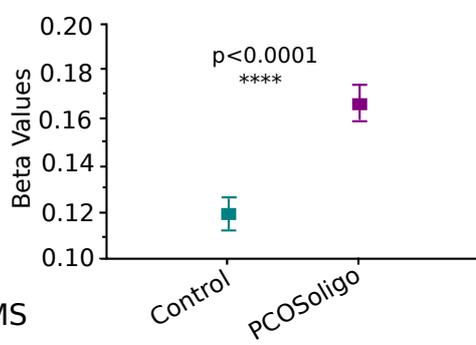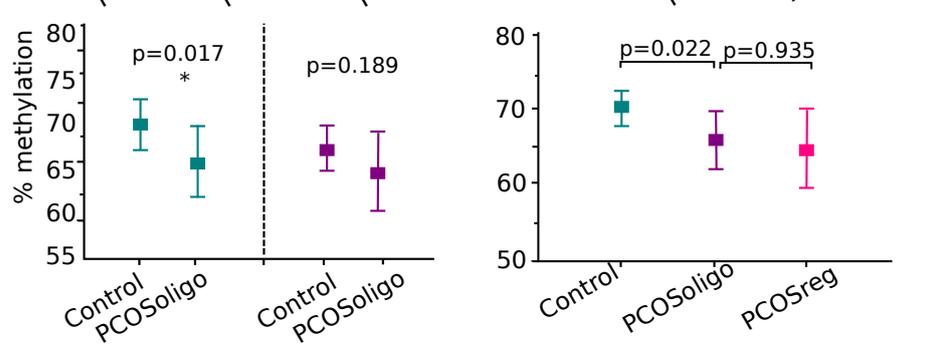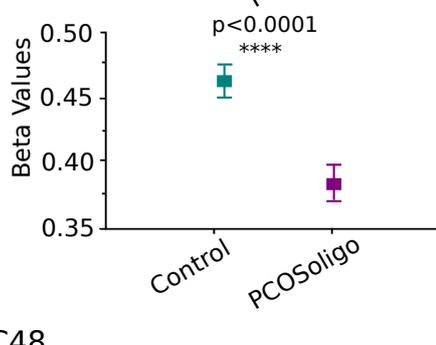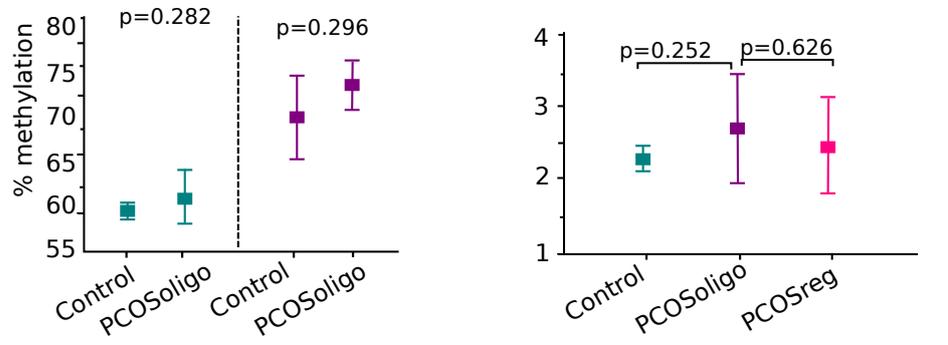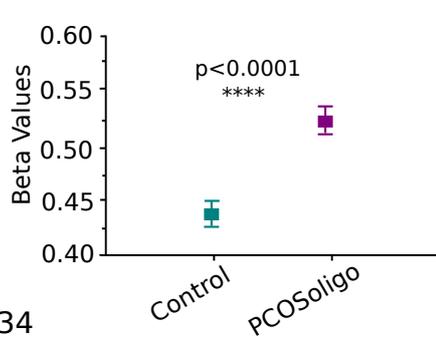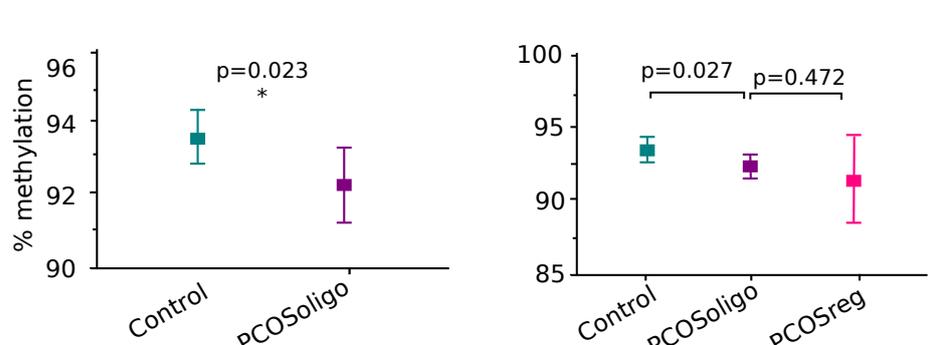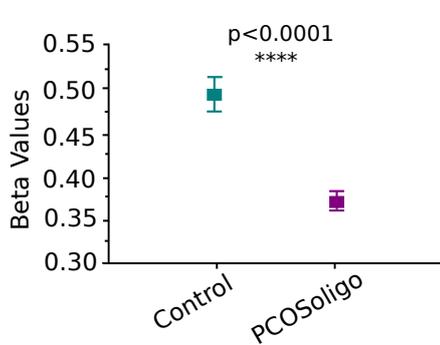
Figure 5
Click here to download high resolution image
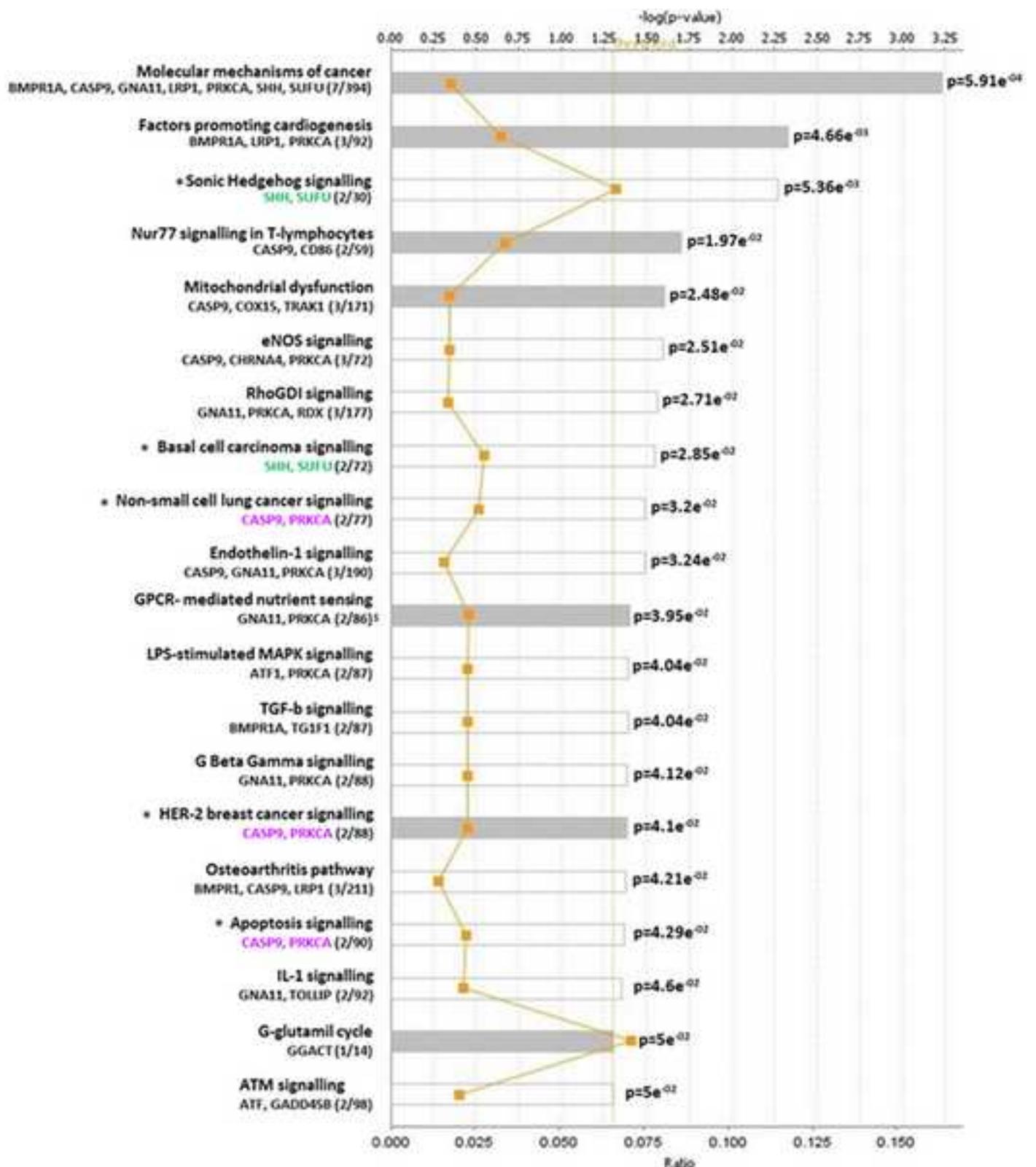
Figure 5

Figure 6

Locus	Reference	Gene	Ancestry	Epic Hits	Associated Genes	Relative Gene Distance
11q.22.1	Day et al. 2015, Chen ZJ et al. 2011, Li et al. 2012	YAP1	European, Han Chinese	2	EXPH5 (**mQTL**), RDX	
12q.13.2	Day et al. 2015, Chen ZJ et al. 2011	RAB5B / SUOX	European, Han Chinese	3	ATF1, LRP1 (**mQTL**), NCKAP5L	
12q.14.3	Day et al. 2015, Chen ZJ et al. 2011, Hayes et al. 2015, Louwers et al 2013	HMGA2	European, Han Chinese	1	HMGA2	
12q.21.2	Hayes et al. 2015	KRR1	European	1	PPFIA2	
19p.13.3	Chen ZJ et al. 2011, Brower et al. 2015	INSR	European, Han Chinese	7	CELF5 (**mQTL**), GADD45B, GNA11, CNN1 (**mQTL**), MUM1 (x2), SLC25A23 (**mQTL**)	
20q.13.2	Chen ZJ et al. 2011	SUMO1P1	Han Chinese	2	CHRNA4, RP5-1030M6.3	
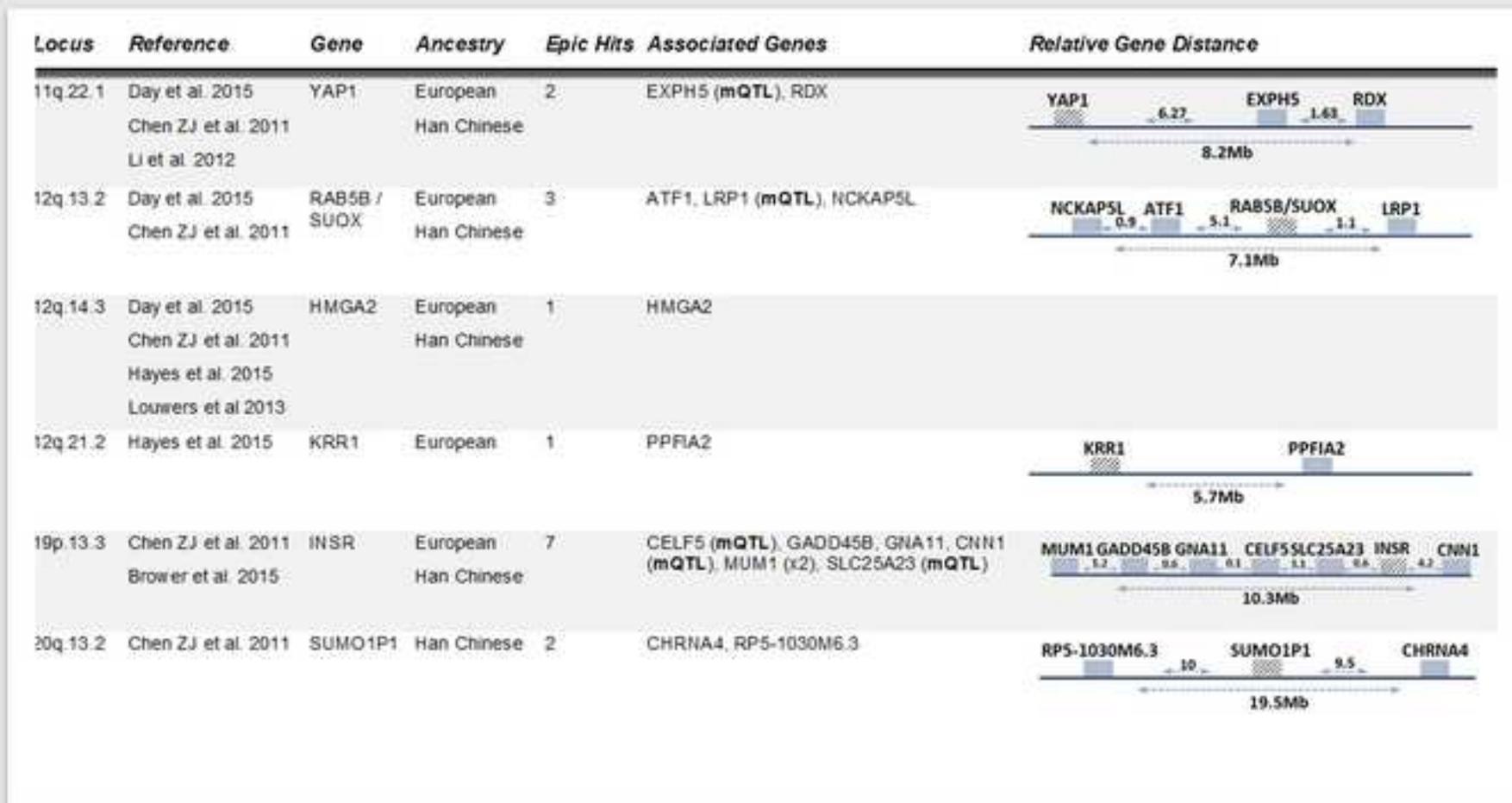
Supplementary Figure S1

Supplementary Table S1

Supplementary Table S2

Supplementary Table S3

Supplementary Table S4

Supplementary Table S5

Authors' roles statement

Eleni Makrinou processed the samples, analysed and interpreted the array-based data, performed, analysed and interpreted the pyrosequencing generated data, prepared Figures and Tables, and wrote the manuscript;

Alexander W. Drong wrote the R scripts for the genome wide analysis, analysed and interpreted the array-based data; T.C. edited the manuscript;

Avigdor Lerner processed samples and offered advice;

George Christopoulos. and Stuart Lavery were involved in sample collection;

Stephen Franks conceived and supervised the study, edited the manuscript;

Kate Hardy conceived and supervised study;

Cecilia Lindgren supervised analysis of the array-based data.