Epigenetic reprogramming of immune cells in women with PCOS impact genes controlling reproductive function

Short Title: Epigenetic programming in immune cells of women with PCOS

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Disclosure statement

The authors declare that they have no competing interests.

Keywords: Polycystic ovary syndrome; DNA methylation; Epigenetics; Inflammation; AMH; Reproduction.

ABSTRACT

Context. Polycystic ovary syndrome (PCOS) is a chronic disease affecting reproductive function and whole-body metabolism. While the aetiology is unclear, emerging evidence indicates that the epigenetics may be a contributing factor.

Objective. To determine the role of global and genome-wide epigenetic modifications in specific immune cells in PCOS compared to controls and if these could be related to clinical features of PCOS.

Design. Cross-sectional study

Participants. Women with (n=17) or without PCOS (n=17).

Setting. Recruited from the general community.

Main Outcome Measure(s). Isolated peripheral blood mononuclear cells were analysed using multi-colour flow cytometry methods to determine global DNA methylation levels in a cell specific fashion. Transcriptomic and genome-wide DNA methylation analysis was performed on T helper cells using RNA-sequencing and Reduced Representation Bisulfite Sequencing.

Results. Women with PCOS had lower global DNA methylation in monocytes (p=0.006), T helper (p=0.004), T cytotoxic (p=0.004), and B cells (p=0.03). Specific genome-wide DNA methylation analysis of T helper cells from women with PCOS identified 5,581 differentially methylated CpG sites. Functional gene ontology enrichment analysis showed that genes located at the proximity of differentially methylated CpG sites belong to pathways related to reproductive function and immune cell function. However, these genes were not altered at the transcriptomic level.

Conclusions. It was shown that PCOS is associated with global, and gene-specific DNA methylation remodelling in a cell-type specific manner. Further investigation is warranted to determine whether epigenetic reprogramming of immune cells is important in determining the different phenotypes of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a metabolic and reproductive disorder with a complex and ill-defined aetiology. It is commonly characterised by increased levels of androgens, inflammatory cytokines, insulin resistance and increased prevalence of sub-fertility [1-3]. Familial clustering of PCOS is well documented, providing evidence for a heritable component of the syndrome [4,5]. However, investigations to identify the single nucleotide polymorphisms (SNPs) that may underpin the genetic basis of PCOS have been inconclusive despite recent advances in the field from genome-wide association studies (GWAS) [6-9].

Emerging evidence suggests a role for altered epigenetic programming in the aetiology of PCOS [10-14]. Genome-wide analysis of transcriptomic, and DNA methylation status have been conducted in adipose tissue and skeletal muscle revealing tissue-specific epigenetic, and transcriptomic differences between women with and without PCOS [13,14]. In adipose tissue, DNA methylation analysis revealed aberrant patterns in pathways involved in adipogenesis, inflammation, glucose regulation and energy metabolism, and these DNA methylation sites corresponded with 30 differentially expressed genes [13]. Similarly, in skeletal muscle, transcriptomic analysis found significantly enriched pathways in immune function, and disease [14]. Finally, both adipose tissue and skeletal muscle showed inflammation as a significantly enriched pathway. This is important because immune cells play a critical role in metabolism, reproductive function, are the main source of inflammatory cytokines and are known to be epigenetically regulated [15-19]. These studies provide important insights into the potential contribution of epigenetic changes to the development of PCOS and rationale for further investigation into epigenetic changes in other cell types that may be affected in PCOS, such as immune cells. In both animal PCOS models, and women with PCOS, altered immune

responses, and inflammatory markers have been found in various ovarian tissues, suggesting a role in the pathophysiology of the reproductive features of PCOS [20-22]. Impairment of immune cell function and the infiltration of these cells in insulin sensitive tissues has been shown to cause metabolic impairment and contribute to the onset of type 2 diabetes mellitus (T2DM) [23,24]. Thus, epigenetic remodelling of immune cells can be associated with impaired immune function and contribute to metabolic dysfunction in PCOS [15,25,26].

Only one study has investigated the contribution of global epigenetic changes in immune cells from women with PCOS [27]. In this study, they measured global DNA methylation (i.e. total, non-site-specific methyl-cytosine abundance) in a mixed population of immune cells known as peripheral blood mononuclear cells (PBMCs) from women with or without PCOS and failed to find any difference [27]. There is increasing evidence supporting that epigenetic changes that occur within specific cell subtypes could be masked by analyses of whole blood [28]. Therefore, cell-type specific global, and genome-wide methylation analysis may reveal a potential role of epigenetics in the immune system especially in PCOS[28-30]. In the present study we hypothesised that specific immune cell populations will present a different DNA methylation profile in women with PCOS compared to women without PCOS[28,31]. Using global and genome-wide DNA methylation analysis we aimed to explore epigenetic remodelling in specific immune cell-subtypes, most notably in T-Helper cells.

Materials and methods

Study population

Premenopausal women *(n=34)* aged between 18-45 years with or without PCOS were recruited from the local community (i.e. a non-clinical population). All participants provided informed written consent. Women with PCOS were confirmed by an endocrinologist (SS or AJ) to have

features consistent with the Rotterdam diagnostic criteria based on participant's previous medical records. The Rotterdam criteria was used for confirmation of PCOS with two of the following (i) oligo- or anovulation (ii) clinical (hirsutism and acne) and/or biochemical hyperandrogenism (iii) polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism [32,33]. Women without PCOS had no features of PCOS. Exclusion criteria were pregnancy, smoking, T2DM, known cardiovascular disease, asthma and medications affecting endpoint measures including: hormonal contraceptives, insulin sensitising drugs, anti-inflammatories and anti-androgens. This study was approved by the Victoria University Human Research Ethics Committee (HRE 14-138).

Clinical measures

All clinical measures were collected in the morning after an overnight fast. For women without PCOS, testing was conducted in the early follicular phase of the menstrual cycle (2-7 days after beginning menses). All women with PCOS had irregular cycles and therefore the testing was conducted in early follicular phase when possible.

Lifestyle monitoring

In the seven days preceding the trial, participants recorded their dietary intake *via* a 7-day food diary. Food diaries were analysed by FoodWorks® (Xyris) for the major food groups (grains, fruit, vegetables, protein and dairy), total energy (macronutrients) and fat ratios. Moderate-to-vigorous physical activity was measured via an ActiGraph (GTX3+) accelerometer (Actigraph). Participants wore the monitor on their right hip during waking hours for seven days, except for bathing and swimming. Data was downloaded in 1-minute epochs and non-wear time was defined as at least 20 minutes of zero counts. Daily moderate-to-vigorous physical activity was defined as all wear-time minutes and was calculated based on the

Freedson accelerometer age-cut points [34]. Self-reporting physical activity was measured via the International Physical Activity Questionnaire (IPAQ).

Anthropometric assessment

Height and weight were measured to calculate body mass index (BMI): [body weight (kg)/ height squared (m^2)]. Waist and hip circumference measurements were recorded [35]. Fat mass, abdominal fat mass and fat free mass were measured by dual-energy x-ray absorptiometry (DXA): [GE Lunar iDXA] and analysed by a qualified DXA operator (DH).

Fasting blood samples

A sterile polyethylene catheter was inserted into the antecubital vein and baseline blood samples were collected. Plasma and serum were collected in the appropriate blood tubes via centrifugation and stored at -80ºC until analysis. Blood for PBMCs isolation was collected in three acid citrate dextrose tubes (BD) and left at room temperature until ficoll gradient separation on the same day as described below [28].

Oral glucose tolerance test

After collecting fasting baseline blood samples, participants then underwent an oral glucose tolerance test (OGTT) via ingestion of a 75g glucose drink over a 5 minute period with blood samples collected at 30, 60, 90 and 120 minutes [36]. Blood glucose in plasma was measured on the day by using an automated analyser (YSI 2300 STAT Plus). Blood cell counts were measured on the day by haematology automated analyser (Sysmex).

Biochemical analysis

154 The following assays were completed in house at Victoria University. Plasma insulin
155 concentration was determined by radioimmunoassay according to manufacturer instructions
156 with an intra and inter variability of 4.2% and 3.8% respectively (HI-14K, EMD, Merck
157 Millipore). Results from the OGTT were used to determine both insulin sensitivity and
158 resistance using area under the curve for plasma insulin and glucose concentrations and the
159 homeostasis assessment (HOMA) indices of insulin sensitivity calculated as (fasting insulin x
160 fasting glucose/22.5) [36]. Interleukin-6 (IL-6) was measured using a high sensitivity ELISA with
161 an intra assay variability of 7.6% (ab46042, Abcam). S-adenosylmethionine (SAM) was
162 determined by competitive enzyme immunoassay and had an intra assay variability of 8.8%
163 (STA-672, Cell Biolabs).
164
165 The following assays were completed in the accredited pathology laboratory at Monash Health,
166 Australia. Lipid profiles (Cholesterol, Triglycerides, LDL and HDL) were quantified by
167 automated enzymatic methods (Architect C18000 analyser), high sensitivity C reactive protein
168 (hs-CRP) by a highly sensitive Near Infrared Particle Immunoassay using the Beckman Coulter
169 Synchron LX system Chemistry Analyser (Beckman Coulter). Folate was determined by the
170 Access/DXI Folate assay which is a competitive binding receptor assay performed on the
171 Beckman Coulter Unicel DXI 800 (Beckman Coulter). Homocysteine was measured using a
172 Beckman Coulter Synchron DXC800 system Chemistry Analyser (Beckman Coulter). AMH
173 was analysed using an enzyme-linked immunosorbent assay (A16507, Immunotech, Beckman
174 and Coulter Company). The sex hormone binding globulin (SHBG) assay was performed using
175 a sequential two-step immunoenzymatic ('sandwich') assay carried out on a Beckman Coulter
176 Unicel DXI 800 (Beckman Coulter). Testosterone was measured using high performance liquid
177 chromatography–mass spectrometry (HPLCMS/MS) method using a liquid sample extraction
178 (AB Sciex Triple Quad 5500 LC/MS/MS system). Free androgen index (FAI) was calculated

as (total testosterone x 100)/SHBG. Free testosterone (fT) was calculated by the Södergard free Testosterone calculation [37].

PBMCs isolation

PBMCs were isolated by ficoll gradient as previously described in [28] with some modifications. After centrifugation (1200 x g for 20 mins, brake off) and removal of plasma, the cell suspension was diluted in Roswell Park Memorial Institute medium (RPMI, Thermofisher), supplemented with penicillin/streptomycin and L-glutamine and carefully layered on ficoll in Sepmate™ tubes (Stemcell technologies) in equal volumes and centrifuged. The buffy coat containing PBMCs was collected, washed with RPMI, and resuspended in autologous plasma. Cells were counted on the automated cell counter (TC20™ automated cell counter, Bio-Rad) before being frozen in 10% Cyropreservent DMSO in RPMI overnight in a controlled rate freezing container (CoolCell®, Biocision) and transferred to liquid nitrogen the following day for long-term storage.

Global DNA methylation

Global methylation in PBMCs was quantified as previously described [28]. Briefly the cells were stained with an antibody cocktail of anti CD3-phycoerytrin conjugated, CD8- and CD14-peridinin chlorophyll conjugated, CD4- and CD19-allophycocyanin conjugated (Becton-Dickinson). Cells were then further stained with anti-5-methylcytosine (5meC, AbD serotec, Bio-Rad) or with its associated isotope control (Mouse, IgG1, BD) labelled with Alexa Flour 488 according to manufacturer's instructions (Zenon Alexa Flour 488 Mouse IgG1, Molecular probes, Life Technologies). Cells were incubated (20 min in the dark at room temperature) and then run immediately on the flow cytometer (FACS Calibur, BD). A specific gating strategy (supplemental figure 1) was used to separate the different cell populations and the median

fluorescence intensity (MFI) was measured and normalised by the MFI from the isotope controls for each cell population. Data were analysed using FlowJo version 10 (Tree Star) and Cytobank (Cytobank Inc).

Cell sorting

Cryopreserved PBMCs were thawed and immediately resuspended in RPMI before being washed and stained with anti-CD3 (APC), anti-CD4 (PE), anti-CD8 (Brilliant Violet or BV 510), anti-CD19 (BV421), anti-CD20 (PE-Cyanine or Cy7) and anti-CD14 (APC). All antibodies were sourced from BD Biosciences (supplementary table 1[38]). The stained PBMCs were washed twice in sort buffer [PBS 1% BSA, 25mM HEPES (pH=7.0), 1mM EDTA] before being sorted into four populations (monocytes, T helper, T cytotoxic, and B Cells) using the FACS-Aria (BD).

RNA sequencing

Total RNA from T helper cells was extracted using the Qiagen all prep DNA/RNA/miRNA universal kit (#80224, Qiagen) following manufacturer's instructions. Quality of RNA was established using the Agilent RNA 600 Nano kit and Bioanalyser instrument (Agilent Technologies). RNA sequencing was performed according to Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold protocol (Illumina) as previously described [39]. Each library was quantified to ensure optimum cluster densities across every lane of the flow cell using the Qubit dsDNA HS assay kit (Invitrogen). Quality control for base pair size and purity was assessed using the Agilent High Sensitivity DNA chip and Bioanalyser instrument (Agilent Technologies). Each library was diluted to 1nM before being pooled and measured on the Illumina Next Seq 500 (Illumina).

Reduced representation bisulphite sequencing (RRBS)

Genomic DNA was extracted from T helper cells using the Qiagen all prep DNA/RNA/miRNA universal kit (#80224, Qiagen) following manufacturer's instructions. RRBS was performed using Diagenode Premium RRBS Kit (#C02030033, Diagenode) following manufacturer's instructions. Each library was quantified using the Qubit dsDNA HS assay kit (Invitrogen) and quality controlled for base pair size and purity using the Agilent High Sensitivity DNA chip and Bioanalyser instrument (Agilent Technologies). Genome-wide DNA methylation of T helper cells was measured on the Illumina Next Seq 500 (Illumina).

Transcriptomic analysis

RNA-seq raw reads were aligned to human genome (hg38) using STAR [40] and gene coverages were computed by featureCounts [41] using Gencode annotation [42]. The ribosomal RNA counts were excluded from the downstream analysis. Libraries with less than 15 million assigned reads were removed from analysis. A generalised linear model ($y \sim 0 + disease$) was fitted for disease factor by using DEseq2 pipeline [43]. Genes with a false discovery rate (FDR) below 0.1 were considered differentially expressed.

Approximately 15 million reads/sample were assigned to genes with 38 genes surviving the expression threshold ($q<0.1$). Molecular functions and biological processes were established from the Universal Protein Resource (UniProt) which is a comprehensive resource for protein sequence and annotation data. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) database investigated the interaction of molecular pathways that were associated with the identified differentially expressed genes. This provided an *in-silico* indication of the molecular pathways that may be affected in the T helper cells of women with PCOS.

RRBS analysis

RRBS reads were processed with the 'rrbs' setting of Trim Galore v0.3.7 and Cutadapt v1.4.2. Processed reads were mapped to hg38 followed by derivation of CpG methylation using Bismark [44]. Mappings to Y chromosome was removed for the differential methylation analysis. Differential methylation analysis was conducted on site and region level according to the sample groups (PCOS v CON) by using RnBeads pipeline [45]. For each library, SNP-enriched sites were removed and sites with less than 10 counts were masked. Gene ontology (GO) enrichment analysis was conducted to determine significant GO terms using a hypergeometric test and a rank cut-off applied for the top 100 best ranking regions in RnBeads pipeline.

Statistical analysis

Data were analysed using IBM SPSS Statistics, version 22 (Armonk, NY). Baseline characteristics are presented as mean ± SD or median (IQR), when data were skewed. The baseline characteristics between PCOS and control groups were compared using student t-tests if data was normally distributed. Data were tested for normality using the Shapiro-Wilk test and when deemed non-normal the Mann Whitney test was used to compare the baseline characteristics. Student t-test were used to examine the difference in global DNA methylation between women with and without PCOS. Statistical significance was accepted when $p<0.05$. To get insight into the relationship between clinical perturbations and DNA methylation in blood, we performed spearman correlation analyses in women with or without PCOS for each population of PBMCs, assessing the association between body composition, physical activity, dietary intake and hormones with global DNA methylation. After adjusting for multiple comparisons statistical significance was accepted when false discovery rate (FDR) $q\leq0.1$. The datasets generated and/or analysed during the current study are available in the NCBI's Gene Expression Omnibus (GEO) database through the GEO series accession number GSE130582[46].

Results

Clinical and biochemical characteristics

All 17 women with PCOS had irregular cycles or amenorrhea; 14 had clinical or biochemical hyperandrogenism, and 15 had polycystic ovaries on ultrasound. Women without PCOS did not display any of the three clinical features detailed by the Rotterdam criteria [33,47]. Detailed clinical characteristics of the 34 women (n=17 PCOS; n=17 without PCOS) who participated in study are reported in Table 1. There were no differences in age, height, weight, body mass index (BMI), markers of body composition, physical activity, energy, and macronutrient intake between women with or without PCOS (Table 1). There were also no differences in the lipid profile, circulating methyl donors (SAM or homocysteine), cytokines (interleukin or IL-6 and high sensitivity C - reactive protein), or a difference in the fasting and the postprandial response to the oral glucose tolerance test (OGTT) and homeostatic model assessment for assessing insulin resistance (HOMA-IR) (Table 2). There was a tendency for folate to be higher in women with PCOS ($p=0.08$). Women with PCOS showed a higher area under the glucose curve ($p=0.02$) during the OGTT compared with women without PCOS, but there was no difference observed for area under the insulin curve between groups (Table 2). Women with PCOS had higher levels of free testosterone (fT) ($p<0.01$), free androgen index (FAI) ($p<0.01$) and anti-müllerian hormone (AMH) ($p<0.01$) (Table 2).

Global DNA methylation

Global DNA methylation analysis of the individual immune cell populations was performed using an antibody that detects methylated cytosine bases coupled with FACS detection [28]. We identified hypomethylation in monocytes ($p=0.006$), T helper ($p=0.004$), T cytotoxic ($p=0.004$), and B Cells ($p=0.03$) in women with PCOS compared to women without PCOS

(Figure 1). Furthermore, systemic AMH concentration was associated with global DNA hypo-methylation in T helper (r_s= -0.428, q=0.078), but not in T cytotoxic cells, B cells or monocytes (Table 3). Subset analysis revealed that the AMH associated hypomethylation in T helper cells was only observed in women with PCOS (p=0.035). fT was also associated with DNA hypomethylation in T helper (r_s= -0.381, q<0.087), but not T cytotoxic cells, B cells or monocytes (Table 3). Collectively, our results show a cell-type specific alteration of global DNA methylation in immune cells in PCOS and suggest a correlation with the dysregulated ovarian hormone and hyperandrogenism as indicted by associations with AMH and fT respectively.

DNA methylome of T helper cells

To further explore the changes in the methylome of immune cells from women with PCOS, we investigated genome wide methylation levels in sorted T helper cells. We identified 5,581 CpG sites differentially methylated in women with PCOS in T helper cells. Of these sites, only 13 were located within promoter regions or gene bodies (8 promoters and 5 gene bodies, Table 4). Gene enrichment analysis of the genes harbouring differentially methylated cytosine's identified gene ontology (GO) terms related to T cell function, and reproductive function including; female pregnancy, response to prolactin, regulation of ovarian follicle development, progesterone receptor signalling pathway, male sex determination and response to steroid hormone (Supplementary Table 2-5[38]). Thus, our results indicate that genes regulating reproductive function are epigenetically remodelled at in specific immune cells.

Transcriptomic analysis of T helper cells

To determine if the specific DNA methylation signature we identified in T helper cells is linked to transcriptional changes, we investigated gene expression via RNA sequencing. We found 37

genes differentially expressed between women with PCOS (Figure 2), compared to those without PCOS with 33 down-regulated and 4 were upregulated (Table 5). Surprisingly, none of the differentially expressed genes showed changes in DNA methylation as identified by reduced representation bisulphite sequencing (RRBS) analysis. Interestingly a large portion of the differentially expressed transcripts (n=17) were long non-coding RNAs (lncRNA) including pseudogenes, long intergenic non-coding RNA (lincRNA) and Y RNA. The remaining 20 transcripts were protein-coding and related to inflammatory and immune cell function. KEGG analysis revealed that the cyclic guanosine monophosphate (cGMP) signalling and Bone morphogenetic pathway (BMP) signalling pathways were functionally enriched. BMP signalling was upregulated, whereas cGMP signalling was downregulated in women with PCOS. Taken together, our results indicate that epigenetic remodelling of genes related to reproductive function in T helper cells does not seem to be functional at the transcriptomic level in women with PCOS.

Discussion

Here, we report that immune cells from women with PCOS have a distinct epigenetic profile, at both *global* and *gene-specific* level. On the global level we found hypomethylation in all PBMCs sub-types. In the specific T-lymphocyte population, the differentially methylated genes that we identified were enriched for gene ontology terms related to T cell function, but also reproductive function, suggesting a role of epigenetic reprogramming in the reproductive defects associated with PCOS.

Global DNA Methylation

At the cell-type specific level, PBMCs from women with PCOS display distinct global DNA methylation levels compared to women without PCOS. Only one previous study reported that

global DNA methylation levels were not different in the total PBMCs fraction from women with PCOS [27]. While we have not measured the global methylation of the total PBMCs fraction in the present study, differences at the cell-type specific level may not be detected when investigating global DNA methylation at the whole PBMC level. This would be consistent with a previous study showing no difference in global DNA methylation in total blood fractions from subjects with type 2 diabetes while global DNA methylation was altered in specific immune cell populations [28]. Further consolidating the importance of measuring global DNA methylation in immune cells in a cell-type specific manner.

Methylation of DNA is under the control of the one-carbon metabolism notably the methyl donor SAM, which is a cofactor necessary for the transfer of a methyl group to a cytosine base in DNA [48]. To gain insight into the potential relationship between altered global DNA methylation levels in immune cells in PCOS and folate metabolism, we measured key one-carbon metabolites: SAM, homocysteine and folate but found no differences. The lack of association between circulating one-carbon metabolites and global DNA methylation levels in immune cells from women with PCOS suggests that methyl donors may not be the primary driver of the global DNA hypomethylation observed in PCOS. Alternatively it would be interesting to investigate whether the enzymes DNA methyltransferases (DNMTs) or ten-eleven translocation (TET), that are responsible for the transfer of the methyl group to the cytosine base or removal of methyl group respectively, may play a role in the hypomethylation observed in the immune cell populations [49].

Lifestyle factors such as physical activity and diet have been previously associated with both global, and gene-specific DNA methylation changes [50-52]. Obesity, as measured by BMI, is also associated with distinct DNA methylation patterns [28,31,53,54]. In the present study, we did not

find any associations between global methylation and BMI, habitual physical activity or diet (total energy) in any of the cell subsets. These data do not support a role of adiposity, physical activity or caloric intake on the altered global DNA methylation in PBMCs from women with PCOS. Interestingly, we found a negative association between global methylation in T helper cells and circulating levels of AMH. This relationship is only present in women with PCOS who have elevated circulating levels of AMH, suggesting a mechanistic association of this hormone in PCOS and DNA methylation changes in T helper cells. AMH is a member of the transforming growth factor beta (TGF-β) ligand superfamily that is predominantly present in ovaries in healthy women but released into the circulation at elevated levels in PCOS [2,55-60] although there is little literature indicating whether it is a cause or consequence. AMH has been found to cluster with many clinical markers of PCOS including positive correlations with LH, and androgens and negatively with FSH, and glucose levels [59,60]. Furthermore, in women with PCOS, it has been shown that AMH disrupts folliculogenesis by decreasing sensitivity to FSH and thereby inhibiting follicle recruitment, and growth which can result in increased number of pre-antral and antral follicles and the PCO morphology [61,62]. It has also been suggested that circulating AMH may have a functional role outside of the reproductive system [63]. Interestingly, in immune cells, members of the TGF-β ligand superfamily are potent regulators of T cell activation and differentiation and control a variety of regulatory epigenetic signals such as chromatin remodelling, histone modification and DNA methylation [64-66]. Based on our observations and considering the literature, we hypothesise that AMH participates in the epigenetic reprogramming of some subpopulations of PBMCs in women with PCOS warranting further research.

Here we identified a negative correlation between global methylation in T helper cells and fT. In women with PCOS, fT is elevated and is used to diagnose hyperandrogenism in the

syndrome[32]. Elevated androgens in animal models are associated with reproductive dysfunction (oligo-anovulation, menstrual disturbances and sub-fertility) that is observed in PCOS [61,67-70]. Androgens have immunomodulatory effects, and elevated androgens are associated with altered immune function that likely impacts reproductive function [71,72]. Medawar[73] identified the importance of the immune system in reproduction with further studies identifying the importance in the frequencies of T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T (Treg) cells in maintenance of normal ovarian function, and menstrual cycles [73-76]. Interestingly Th1/Th2/Th17 imbalances have been identified in women with PCOS [76-78]. The differentiation of T cells and the ratios of Th1/Th2/Th17/Treg cells appear to be modulated by androgens [72,79-81]. Furthermore, the differentiation of T cells are also modulated by epigenetic mechanisms [15,82,83] and this may be the case in PCOS [72]. Altogether our data complements previous studies indicating the elevated fT was associated with differences in the methylome profile in T helper cells in women with PCOS. Whether hyperandrogenism modulates the epigenome of T helper cells and result in changes to frequency of Th1/Th2/Th17/Treg cells and the reproductive dysfunction in PCOS would be an interesting avenue to explore.

DNA methylome, and transcriptomic analysis of T helper cells

We also investigated genome-wide DNA methylation in T helper cells at the single nucleotide resolution and identified differentially methylated cytosine bases at the proximity of genes controlling reproductive function. Specifically, our gene ontology analysis returned terms such as female pregnancy, regulation of ovarian follicle development, and male sex determination. As the altered DNA methylation was present at genes controlling reproductive function in immune cells, this suggests that epigenetic changes may not be tissue specific. Instead, these methylation patterns may occur in multiple tissue-types that are exposed to specific extra-

cellular stimuli (possibly elevated AMH levels or elevated androgens), with such exposure potentially occurring *in-utero* or as a result of the development of PCOS over the lifespan [84-86]. With epigenetic reprogramming of genes being related to reproductive function it would be worthwhile investigating other tissues, such as the ovary or neuro-endocrine tissues, where the appropriate transcriptional activators and co-activators are expressed [87,88]. This would provide insights into the role of epigenetic reprogramming at genes controlling reproductive function on ovarian function and sex hormone imbalances in women with PCOS.

We found no overlap between the transcriptomic, and DNA methylation data. This suggests that the shift in epigenetic signature could be related to differences in the proportions of T-helper subpopulations (T helper 1, T helper 2, T helper 9, T helper 17, follicular T helper, regulatory, naïve, effector, and memory T cells) [89-91]. Previous literature has shown in women with PCOS an increased frequency of the pro-inflammatory Th1 population and a reduced frequency of the in the anti-inflammatory Th2 population [76,78,79]. While we were unable to confirm the frequency of T helper subpopulations, the gene ontology pathway analysis identified differential methylation in the activation and proliferation of different T helper subpopulations suggesting that women with PCOS may indeed have different proportions of the T helper sub-types (supplementary tables 2-5[38]).

To further consolidate this hypothesis, the transcriptomic analysis of the differentially expressed genes in T helper cells identified pathways that relate to T cell activation and differentiation. Of interest, KEGG analysis revealed that the Bone Morphogenic Protein (BMP) signalling pathway was upregulated in women with PCOS, and BMP, like AMH, is a member of the TGF-β ligand superfamily [92]. The elevated circulating AMH found in our women with PCOS may explain the upregulation of BMP signalling in T helper cells *via* activation of the

BMP receptors (BMPR). AMH intracellular signalling works via the BMPR through Smad 1/5/8 pathway, that assemble into a complex with Smad 4 (Co-Smad) and translocate into the nucleus activating a range of genes dependent on the cellular context [93-95]. In T cells, the BMP signalling generally regulates activation and differentiation of circulating naïve immune cells, the proliferation of T helper 9, 17, and memory T cells [95,96]. Despite the lack of quantification of T Helper cells frequency in women with and without PCOS, our data provides a potential mechanism by which the upregulated BMP signalling pathway could impact the Th1/Th2 balance but warrant further studies [93,97].

Several differentially expressed genes were associated with inflammation/inflammatory status (Table 5) in women with PCOS compared to those without. This supports the role of inflammation/inflammatory status consistent with previous studies [98], where ovulation requires an appropriate inflammatory reaction [18]. In addition, circulating immune cells and the cytokines they produce are also involved in ovarian function [16,99,100]. Suggesting that the epigenetic marks in our study can alter the transcriptome towards a pro-inflammatory T helper phenotype and suppress ovarian function.

Many of the differentially expressed genes identified from our transcriptomic analysis were non-coding, with the majority being long non-coding RNAs (lncRNAs) including; pseudogenes, anti-sense RNA and long intergenic non-coding RNAs (lincRNA). LncRNAs are widely expressed and regulate gene expression particularly during development, differentiation and activation of immune cells [101] and have been implicated in the co-morbidities associated with PCOS including T2DM [102,103] and inflammatory disorders [104]. There is also evidence to suggest that lncRNAs can act as a molecular scaffold for epigenetic modifications including DNA methylation and histone modifications [105,106]. Indeed, the lack of overlap in our analysis

between DNA methylation and gene expression suggests that other mechanisms such as lncRNA modulating the transcriptomic in PCOS.

The strength of this study is that this cohort was a well characterised, community recruited group of women with and without PCOS, who were otherwise healthy. We were adequately powered to answer our *apriori* aims with post hoc analysis revealing that we were powered on average 93% for all immune cells populations and had large effect sizes of approximately 0.90 [107]. We acknowledge that there is a need for more definitive research into the molecular mechanisms behind each of the different the phenotypes of PCOS. While in the present study we were unable to confidently address the potential epigenetic differences between phenotypes in PCOS, we were able to explore the heterogeneous immune cell population and the unique role these may play in PCOS. These findings provide novel avenues for future research in PCOS including studying the role of methylation in specific immune cells in determining the different phenotypes in PCOS.

In conclusion, our study in a small, yet well-characterised cohort of women with and without PCOS demonstrates novel epigenomic insights into PCOS. The specific epigenetic reprogramming of genes involved in reproductive function in immune cells from women with PCOS is intriguing and may indicate a role for epigenetic factors in the reproductive dysfunction and sex hormone imbalance associated with PCOS. Finally, we found that global DNA methylation in T helper cells is negatively associated with circulating levels of AMH and fT, suggesting the importance of the cellular milieu (elevated circulating androgens and AMH) in the programming of T helper cells in PCOS and warrants further investigation.

Acknowledgements

We would like to acknowledge Dr Anju Joham (AJ) and Dr Soulmaz Shorakae (SS) for confirming diagnosis of women with PCOS. Professor Helena Teede for the critical review of study design and expertise. Dr Sarah Voisin for assistance with statistical analysis.

Data Availability

All data generated or analysed during this study are included in this published article or in the data repositories listed in References

Funding

This work was supported by the Novo Nordisk Foundation Centre for Basic Metabolic Research, an independent research centre at the University of Copenhagen partially funded by an unrestricted donation from the Novo Nordisk Foundation and the NHMRC Centre for Research Excellence in PCOS. These funding bodies did not have a role in study design and have no role in the implementation of the study.

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Legends

Table 1: Anthropometric data and lifestyle characteristics.

Table 2: Metabolic and reproductive profile of participating women with and without PCOS.

Table 3: Spearman correlations of *apriori* co-variates with global DNA methylation in T helper cells, T cytotoxic cells, monocytes, B cells across all participants.

Table 4: Genes and promoters carrying differentially methylated CpGs in T helper cells from women with PCOS compared to women without PCOS.

Table 5: Differentially expressed genes in T helper cells from women with PCOS compared to women without PCOS.

Figures

Figure 1: Lower 5-methylcytosine levels in monocytes, T helper, T cytotoxic and B cells in women with PCOS. Comparison of 5-methylcytosine median fluorescence intensity (MFI) between women with PCOS (square) and women without PCOS (circle). MFI normalised by the MFI from the isotope control. Significantly different from control *$p<0.05$ **$p<0.01$

Figure 2: Volcano plots representing differentially expressed genes in T helper cells of women with PCOS compared to women without PCOS. Red circles highlight differentially expressed genes. False discovery rate (FDR) $q<0.1$.

Tables

Table 1: Anthropometric data and lifestyle characteristics.

Clinical features	Sample Size n	Without PCOS Mean ± SD Median [IQR]	With PCOS Mean ± SD Median [IQR]	p
General characteristics				
Age (years)	34	30.1 ± 6.6	28.9 ± 4.8	p=0.6
Height (cm)	34	165 ± 7.2	162 ± 4.7	p=0.3
Weight (kg)	34	70.5 ± 16.8	71.5 ± 18.2	p=0.9
BMI (kg/m^2)	34	25.5 ± 5.4	26.6 ± 6.9	p=0.6
WHR	32	0.85 [0.8, 1.0]	0.80 [0.8, 0.9]	p=0.2
Body fat (%)	31	32.3 [25.9, 40.5]	37.7 [31.8, 41.8]	p=0.3
Physical activity				
PA (IPAQ-mins.week)	31	172 [15.0, 427.5]	165 [40.0, 255.0]	p=0.6
PA (Accel-mins.day)	30	47.0 [45.5, 48.5]	47.0 [46.0, 50.5]	p=0.6
Food intake				
Energy (g)	31	8269 [7033, 11402]	7744 [5509, 8885]	p=0.2
Saturated fats (g)	31	28.0 [23.0, 50.0]	21.5 [17.2, 30.3]	p=0.07
Carbohydrates (g)	31	198 [157, 278]	171 [144, 220]	p=0.2
Sugar (g)	31	70.0 [56.5, 118]	67.5 [51.0, 123]	p=0.6

BMI, body mass index; WHR, waist-to-hip ratio; IPAQ, International Physical Activity Questionnaire; MVPA; Moderate Vigorous Physical Activity; PA; physical activity.

Table 2: Metabolic and reproductive profile of participating women with and without PCOS.

Clinical features	Sample Size n	Without PCOS Mean±SD Median [IQR]	With PCOS Mean±SD Median [IQR]	p
Lipid profile				
Cholesterol (mmol/L)	30	4.2 ± 0.7	4.4 ± 0.7	p=0.5

Triglycerides (mmol/L)	30	0.6 [0.6, 0.9]	0.8 [0.5, 1.0]	p=0.6
HDL(mmol/L)	30	1.5 ± 0.3	1.5 ± 0.3	p=1.0
LDL (mmol/L)	30	2.5 ± 0.6	2.4 ± 0.7	p=0.6
OGTT				
Fasting glucose (mmol/L)	34	4.9 [4.8, 5.4]	4.9 [4.6, 5.3]	p=0.4
Fasting insulin (pmol/L)	34	72.8 [56.2, 89.4]	68.5 [47.2, 102.4]	p=0.9
2hr glucose (mmol/L)	34	4.75 [4.5, 5.4]	5.5 [4.6, 5.8]	p=0.2
2hr insulin (pmol/L)	34	278 [207, 399]	309 [165, 737]	p=0.5
AUC Glucose (mmol/L)	33	665.8 ± 75.4	783.7 ± 167.8	p=0.02
AUC Insulin (mmol/L)	33	48012 ± 18367	66180 ± 40350	p=0.11
HOMA	34	2.65 [2.0, 3.2]	2.41 [1.7, 4.4]	p=0.9
Reproductive markers				
fT (pM)	34	23.2 [17.7, 31.2]	10.80 [10.3, 14.1]	p<0.001
SHBG (nmol/L)	34	62.1±21.5	63.4±38.4	p=0.9
FAI	34	1.42 [1.4, 2.2]	3.17 [2.2, 5.3]	p<0.001
AMH (pmol/L)	33	18.9 [10.8, 31.1]	48.6 [37.7, 74.6]	p<0.001
Systemic methyl substrate/donors				
SAM (µg/mL)	32	7.7 [5.4, 8.3]	6.9 [6.0, 12.8]	p=0.7
Folate (nmol/L)	33	29.9 [23.2, 37.5]	37.3 [23.4, 45.8]	p=0.08
Homocysteine	33	9.0 [6.9, 10.1]	7.1 [6.4, 9.7]	p=0.3
Cytokines				
HsCRP (mg/L)	30	1.3 [0.5, 3.3]	1.4 [0.6, 4.5]	p=0.7
IL-6 (pg/ml)	31	1.8 [1.3, 3.0]	2.1 [1.5, 2.9]	p=0.3
Cell counts				
WBC (µL)	31	5335 ± 1229	5750 ± 1497	p=0.4

OGTT, oral glucose tolerance test; AUC, area under the curve; HOMA, homeostatic model assessment, fT, free testosterone; SHBG, Sex Hormone Binding Globulin; FAI, Free Androgen Index; AMH, Anti-Müllerian Hormone; IL-6, Interleukin-6; HsCRP, High sensitivity C-reactive protein; SAM, S-adenosylmethionine.

Table 3: Spearman correlations of *apriori* co-variates with global DNA methylation in T helper cells, T cytotoxic cells, monocytes, B cells across all participants.

Covariate		Spearman Correlation			
		T helper	T cytotoxic	Monocytes	B Cells
BMI (kg/m^2)	r_s	0.007	0.021	0.140	0.143
	p-Value	0.967	0.905	0.430	0.420
	FDR q-value	0.967	0.900	0.516	0.504
PA (MVPA mins.day)	r_s	-0.139	-0.166	-0.165	-0.213
	p-Value	0.463	0.380	0.383	0.259
	FDR q-value	0.694	0.735	0.5164	0.504
Energy (g)	r_s	0.070	-0.068	-0.069	-0.095
	p-Value	0.708	0.716	0.712	0.612
	FDR q-value	0.846	0.852	0.8546	0.612
AMH (pmol/L)	r_s	**-0.428**	-0.317	-0.286	-0.191
	p-value	**0.013**	0.072	0.106	0.288
	FDR q-value	**0.078**	0.432	0.351	0.504
fT (pM)	r_s	**-0.381**	-0.237	-0.278	-0.175
	p-Value	**0.029**	0.183	0.117	0.330
	FDR q-value	**0.087**	0.549	0.351	0.504
AUC glucose (mmol/L)	r_s	-0.160	-0.124	-0.152	-0.154
	p-Value	0.373	0.493	0.399	0.393
	FDR q-value	0.694	0.735	0.516	0.504

BMI, Body Mass Index; MVPA, Moderate Vigorous Physical Activity; PA; physical activity; AMH, Anti-Müllerian Hormone; AUC, area under the curve. r_s, Spearman's Rho; fT, free testosterone. Significance was considered when false discovery rate (FDR) q<0.1.

845 **Table 4: Genes and promoters carrying differentially methylated CpGs in T helper cells from women with PCOS compared to women**

846 **without PCOS.**

Genes				
Gene symbol	**Gene Name**	**ENSEMBL Gene ID**	**Chromosome**	**Function and gene type**
COX6CP15	*cytochrome c oxidase subunit 6C pseudogene 15*	ENSG00000228092	chr10	pseudogene
SCGB1D4	*secretoglobin family 1D member 4*	ENSG00000197745	chr11	Regulation of chemotactic cell migration and invasion.
NA	*AC025678.3*	ENSG00000279092	chr15	TEC (To be experimentally confirmed) protein tyrosine kinase-involved in T cell signalling and activation
NA	*AP006565.1*	ENSG00000279092	chr18	anti-sense RNA
NA	*AC104301.2*	N/A	chr20	ncRNA
Promoter of genes				
Gene symbol	**Gene Name**		**Chromosome**	**Function and gene type**
WBP11P1	*WW domain binding protein 11 pseudogene 1*	ENSG00000260389	chr2	pseudogene
SCGB3A2	*secretoglobin family 3A member 2*	ENSG00000164265	chr5	receptor-mediated endocytosis

COX6CP15	*cytochrome c oxidase subunit 6C pseudogene 15*	ENSG00000228092	chr10	pseudogene
SCGB1D4	*secretoglobin family 1D member 4*	ENSG00000197745	chr11	regulation of chemotactic cell migration and invasion.
OVCH1	*ovochymase 1*	ENSG00000187950	chr12	serine-type endopeptidase activity, hydrolase activity, metal ion binding, proteolysis
NA	*AP006565.1*	ENSG00000265737	chr18	anti-sense RNA
NA	*AC104301.2*	N/A	chr20	ncRNA
IGLJ2	*immunoglobulin lambda joining 2*	ENSG00000211676	chr22	Immunoglobin

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848 **Table 5: Differentially expressed genes in T helper cells from women with PCOS compared to women without PCOS.**

Down regulated genes in women with PCOS							
Gene name	**Gene symbol**	**ENSEMBL Gene ID**	**log2Fold Change**	**padj**	**Gene type**	**Molecular function**	**Molecular process**
NA	*AC138969.2*	ENSG00000277920	-7.7	0.025	Pseudogene	Unknown	Unknown
ring finger protein 217	*RNF217*	ENSG00000146373	-7.7	0.009	Protein coding	metal ion binding, ubiquitin-protein transferase activity	Unknown
FKBP prolyl isomerase 1B	*FKBP1B*	ENSG00000119782	-7.3	0.029	Protein coding	peptidyl-prolyl cis-trans isomerase activity	Unknown

guanylate cyclase 1 soluble subunit alpha 2	*GUCY1A2*	ENSG00000152402	-7.1	0.062	Protein coding	GTP binding, phosphorus-oxygen lyase activity guanylate cyclase activity, heme binding	Intracellular signal transduction, cyclic nucleotide biosynthetic process, positive regulation of cGMP biosynthetic process, adenylate cyclase activity
caveolae associated protein 2	*CAVIN2*	ENSG00000168497	-6.8	0.037	Protein coding	phosphatidylserine binding, phospholipid binding, protein kinase C binding	plasma membrane tubulation
alcohol dehydrogenase 4 (class II), pi polypeptide	*ADH4*	ENSG00000198099	-6.6	0.062	Protein coding	alcohol dehydrogenase (NAD) activity, all-trans retinal binding, ethanol binding, NAD binding	alcohol catabolic process, cellular aldehyde metabolic process, ethanol oxidation
solute carrier family 5 member 11	*SLC5A11*	ENSG00000158865	-6.6	0.025	Protein coding	transporter activity	transmembrane transport
VWA8 antisense RNA 1 (head to head))	*VWA8-AS1*	ENSG00000278338	-6.5	0.025	lncRNA	Unknown	Unknown
NA	*Z98752.2*	ENSG00000234271	-6.5	0.071	Processed pseudogene	Unknown	Unknown
NA	*AC034102.3*	ENSG00000257449	-6.5	0.044	lncRNA	Unknown	Unknown
NA	*AC253536.6*	ENSG00000272787.1	-6.4	0.009	LincRNA	Unknown	Unknown
atypical chemokine receptor 2	*ACKR2*	ENSG00000144648	-6.4	0.074	Protein coding	C-C chemokine receptor activity,	chemotaxis, inflammatory

						scavenger receptor activity, G-protein coupled receptor activity	response, receptor-mediated endocytosis
keratin 74	*KRT74*	ENSG00000170484	-6.3	0.078	Protein coding	keratin filament binding, structural molecule activity	intermediate filament cytoskeleton organization, cornification, keratinization
NA	*RF00019*	ENSG00000252652.1	-6.3	0.009	Y RNA	Unknown	Unknown
protocadherin gamma subfamily B, 8 pseudogene	*PCDHGB8P*	ENSG00000248449	-6.3	0.070	Transcribed pseudogene	Unknown	Unknown
NA	*LOC101928238*	ENSG00000278611	-6.3	0.074	LincRNA	Unknown	Unknown
transmembrane and coiled-coil domains 2	*TMCO2*	ENSG00000188800	-6.2	0.058	Protein coding	Unknown	Unknown
CELF2 antisense RNA 2	*CELF2-AS2*	ENSG00000237986	-6.2	0.052	lncRNA	Unknown	Unknown
NA	*AC013476.1*	ENSG00000234193.1	-6.1	0.025	lncRNA	Unknown	Unknown
pannexin 3	*PANX3*	ENSG00000154143	-5.9	0.074	Protein coding	wide pore channel activity, gap junction hemi-channel activity	cell-cell signalling, transmembrane transport, cation transport
NA	*RF00019*	ENSG00000202222.1	-5.9	0.029	Y RNA	Unknown	Unknown
NA	*LOC101927851*	ENSG00000238005	-5.2	0.088	LincRNA	Unknown	Unknown
leucine rich repeat containing 9	*LRRC9*	ENSG00000131951	-5.2	0.062	Protein coding	Unknown	Unknown

EXTL3 antisense RNA 1	*EXTL3-AS1*	ENSG00000246339	-5.1	0.040	lncRNA	Unknown	Unknown
KIAA2012	*KIAA2012*	ENSG00000182329	-4.6	0.083	Protein coding	Unknown	Unknown
NA	*AC013643.2*	ENSG00000253875	-4.6	0.062	lncRNA	Unknown	Unknown
cytochrome b reductase 1	*CYBRD1*	ENSG00000071967	-4.4	0.052	Protein coding	ferric-chelate reductase activity, protein binding, oxidoreductase activity, oxidizing metal ions	cellular iron ion homeostasis, response to iron ion, oxidation-reduction process
NA	*AC068620.2*	ENSG00000270147	-4.1	0.078	LincRNA	Unknown	Unknown
NA	*AC243428.1*	ENSG00000229979	-4.1	0.037	Processed pseudogene	Unknown	Unknown
stearoyl-CoA desaturase	*SCD*	ENSG00000099194	-2.7	0.058	Protein coding	stearoyl-CoA 9-desaturase activity, iron ion binding, oxidoreductase activity	lipid metabolic process, fatty acid biosynthetic process, regulation of cholesterol biosynthetic process, oxidation-reduction process
NIMA related kinase 10	*NEK10*	ENSG00000163491	-2.2	0.061	Protein coding	nucleotide binding, protein serine/threonine kinase activity, ATP binding, transferase activity	protein phosphorylation, positive regulation of protein autophosphorylation, positive regulation of MAP kinase activity,

							regulation of ERK1 and ERK2 cascade, regulation of cell cycle G2/M phase transition
thymocyte selection associated family member 2	*THEMIS2*	ENSG00000130775	-0.9	0.009	Protein coding	Protein Binding	immune system process, inflammatory response, cell adhesion, T cell receptor signalling pathway
cAMP-dependent protein kinase inhibitor alpha	*PKIA*	ENSG00000171033	-0.5	0.083	Protein coding	protein kinase inhibitor activity, cAMP-dependent protein kinase inhibitor activity,	negative regulation of transcription by RNA polymerase II, negative regulation of protein kinase activity, regulation of G2/M transition of mitotic cell cycle, negative regulation of protein import into nucleus, negative regulation of catalytic activity

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Upregulated genes in women with PCOS							
Gene name	Gene symbol	**ENSEMBL Gene ID**	log2Fold Change	padj	Gene name	Molecular function	Molecular process
MX dynamin like GTPase 2	*MX2*	ENSG00000183486	0.6	0.0711	Protein coding	nucleotide binding, GTPase activity, protein binding, microtubule binding	mitochondrial fission, immune system process, defence response, response to virus, response to interferon-alpha, innate immune response, mRNA transport
small Cajal body-specific RNA 21	*SCARNA21*	ENSG00000252835	0.6	0.082652	ScaRNA	Unknown	Unknown
histone cluster 1 H3 family member c	*HIST1H3C*	ENSG00000278272	1.4	0.073504	Protein coding	DNA binding, protein binding, cadherin binding, protein heterodimerization activity	chromatin silencing at rDNA, nucleosome assembly, telomere organization, interleukin-7-mediated signalling pathway, negative regulation of gene expression, epigenetic, regulation of hematopoietic stem cell differentiation, regulation of gene silencing
SMAD family member 1	*SMAD1*	ENSG00000170365	3.0	0.099453	Protein coding	RNA polymerase II proximal promoter sequence-specific DNA binding,	MAPK cascade, mesodermal cell fate commitment, osteoblast fate commitment, inflammatory response, transforming growth factor beta receptor signalling pathway, SMAD protein complex assembly, gamete generation, embryonic pattern specification, BMP signalling pathway

						signal transducer activity, downstream of receptor, transforming growth factor beta receptor, pathway-specific cytoplasmic mediator activity, protein homodimerization activity, co-SMAD binding, I-SMAD binding, primary miRNA binding	

Molecular functions and biological processes were established from the Universal Protein Resource (UniProt). False discovery rate (FDR) $q<0.1$.

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