Research Square

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Novel HumanMethylatyion450 Analyses Reveal DNA Methylation Predictors of Future Diabetes Onset in Women With Polycystic Ovarian Syndrome

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Abstract

Aims.

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder characterised by ovarian morphological, systemic biochemical, and menstrual changes. Women with PCOS are at significantly increased risk of raised fasting glucose, impaired glucose tolerance, and diabetes. Recognition of these complications and early intervention are key to good health outcomes. We sought to identify DNA methylation patterns that may predict future diabetes onset in this high-risk PCOS population.

Patients and Methods.

Peripheral blood samples from women with PCOS and women with PCOS who later developed diabetes, were analysed by Illumina HumanMethylation450 BeadChip-arrays. Bisulphite-Pyrosequencing™ was used to validate and confirm array methylation data.

Results.

Array analyses identified 273 differentially methylated CpG loci (≥ 0.2 β-value change) at initial diagnosis of PCOS, between women who did or did not later develop diabetes. 19 of these sites demonstrated differential methylation in the same direction in ≥ five sample pairs. Methylation in three of the candidates (cg11897887, cg02819655, and cg25542007) showed the best concordance with corresponding array β-values, and, most clearly differentiated ‘cases’ from ‘controls’.

Conclusions.

We have identified novel methylation biomarkers that could predict future onset of diabetes in this high-risk population. Use of methylation analyses to identify women who are likely to develop diabetes at diagnosis of PCOS may facilitate timely lifestyle interventions to reduce future morbidity.

Introduction

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder that affects 6–15% of women worldwide, and prevalence is steadily increasing[1, 2]. It is characterised by oligomenorrhea and/or olig- or an-ovulation, androgen excess, and polycystic ovaries (PCO), manifesting as menstrual irregularities, subfertility, hirsutism, acne, metabolic syndrome, and reduced quality of life[1, 2].

PCOS is associated with impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes, all of which are risk factors for cardiovascular morbidity and mortality, and chronic renal disease[3]. Diabetes is approximately six-times more prevalent in women with PCOS than aged-matched non-PCOS

populations, and is the greatest cause of systemic and chronic morbidity in these women[3, 4]. As such, primary prevention of diabetes is key in women with PCOS.

Raised body-mass index (BMI), family history, and ethnicity are risk-factors for developing diabetes in women with PCOS[4, 5]. However, individual risk-stratification is challenging and many women receive sub-optimal lifestyle (e.g. diet, exercise) advice and delayed intervention, with adverse outcomes[1, 5]. Metabolomic and molecular markers have thus far failed to reliably predict development of IGF, IGT or diabetes in women with PCOS[6]. Differential DNA methylation patterns have been found between healthy women and women with PCOS, and between PCOS clinical phenotypes[7]. DNA methylation has also been identified as holding prognostic value in multiple tissue types, including predicting diabetes onset in non-PCOS populations[8].

However, to our knowledge, there are no reports evaluating the prognostic potential of differential methylation in women with PCOS in relation to their risk of developing diabetes. We therefore performed genome-wide ('450K' array) DNA methylation assessment comparing women with PCOS and women with PCOS who later developed diabetes, to determine whether novel potentially clinically useful 'at diagnosis' biomarkers that might guide early intervention and prevent development of diabetes and associated complications, could be identified.

Patients And Methods

All methods and experimentation were carried out in accordance with Keele University guidelines, standard operating procedures and laboratory regulations.

All experimental protocols and study methodology were approved by the University Hospitals of North Midlands NHS Trust Research and Innovation department, and by dedicated Ethics committee (West Midlands (Black Country) Research Ethics Committee 15/WM/0162).

Human blood samples.

Peripheral blood samples were obtained from women with PCOS diagnosed > 12 months prior to sample collection using Rotterdam diagnostic criteria[1]. Repository samples with long-term clinical data enabled identification and comparison of six 'control' (women with PCOS), and six 'case' women with PCOS who developed diabetes (median three years) following sample collection (demographics in **Supplemental Table S1**). Samples were matched by ethnicity, and where possible by age, BMI, smoking status, and co-morbidity. No patient had prior history of endocrine/metabolic disorder, malignancy or systemic chemotherapy.

DNA extraction and bisulphite modification.

Genomic DNA was isolated using AllPrep DNA/RNA Mini kits (Qiagen) then bisulphite-converted using EZ DNA Methylation Gold™ kits (Zymo Research), according to the manufacturers' instructions.

Illumina Methylation Bead-Array Analyses.

Bisulphite-converted DNA from each sample was hybridised to HumanMethylation450 BeadChip arrays (Illumina, San Diego, USA), and processed by Bart's and the London Genome Centre, UK. Array data were processed by GenomeStudio software and the Novel Identification of Methylation Biomarker Lists (NIMBL) platform[9]. NIMBL also assessed internal control data, and performed peak-based correction to compensate for probe-type sensitivity. CpGs where: (i) probe detection p-values were > 0.05, (ii) with missing β-values, or (iii) known to be associated with single nucleotide polymorphisms (SNPs), were excluded.

Pyrosequencing™ of sodium bisulphite-converted DNA.

Validation of array-identified methylation in all samples was performed by Pyrosequencing™ of bisulphite-converted DNA, using PyroMark Q24 Pyrosequencer, PyroMark Q24 Software 2.0 and PyroMark Gold Q24 reagents, according to the manufacturer's instructions.

Results

Initial array data screening identified 273 CpG loci where ≥ 5 of the six matched PCOS 'control' and the PCOS with subsequent-onset diabetes 'case' sample pairs displayed differential methylation (β-value ≥ 0.2) (Fig. 1. Hierarchical clustering and heatmap).

The most promising biomarkers were identified on the basis of differential methylation in the same direction (all either hyper- or hypo-methylated in PCOS control relative to PCOS with diabetes cases) in five or six (out of six) matched pairs. These criteria identified 19 putative methylation biomarkers (cg identifier list in **Supplemental Table S2**). These CpG loci were validated using Pyrosequencing™ in all 12 array samples (Primer sequences in **Supplemental Table S3**). Methylation in three of the candidates (cg11897887, cg02819655, and cg25542007) showed the best concordance with corresponding array β-values, and, upon visual inspection of plotted methylation data, most clearly differentiated cases from controls (Fig. 2).

Discussion

To identify potential clinically useful prognostic DNA methylation biomarkers, we used peripheral blood samples taken from women at their initial diagnosis of PCOS. These samples had accompanying long-term clinical follow-up data (up to 12 years), which enabled direct comparison of 'at-diagnosis' methylation patterns in women with PCOS who did or did not later develop diabetes. We reasoned that the best potential methylation biomarkers would be detectable at this early stage where dietary, exercise, and weight management interventions are most likely to be effective[5].

We used well-established methods of genome-wide methylation assessment (HumanMethylation450 arrays) to identify differences between women with PCOS, and women with PCOS who later developed

diabetes. Similar to previous array-based prognostic marker screening studies, and to reduce the likelihood of false positive targets, we used a ≥ 0.2 β-value difference to define differential methylation[9].

Although there are no striking visual differences between cases and controls in the heatmap of the differentially methylated CpG sites, clustering reassuringly separately grouped the women with PCOS who developed diabetes from the women that did not. The 273 differentially methylated CpG sites and the 19 putative biomarker candidates identified is broadly in keeping with numbers in previous methylation biomarker studies in benign tissue types[10], but is difficult to interpret in the context of lack of similar prior studies in PCOS. Notwithstanding, methylation differences were substantiated by Pyrosequencing™ in our candidates, confirming 'proof of principle' that DNA methylation holds the exciting potential as a prognostic biomarker in PCOS. However, given the number of samples evaluated and the variation in criteria used to define PCOS (and therefore variable population prevalence), we cannot estimate prognostic or predictive values. Furthermore, plotted Pyrosequencing™ data raise the possibility that single nucleotide polymorphisms (SNPs) may be implicated in methylation patterns observed in some of our candidates. Although known SNPs were excluded during array filtering, polymorphic CpG sequences (in all Infinium methylation arrays) necessitate caution in interpretation of results[11].

Whilst it cannot be implied from our data, it is possible that differential methylation at one or more of the sites identified may be contributory to diabetes development in women with PCOS. Further studies in larger patient cohorts are required to confirm our initial findings, evaluate the possible importance of SNPs in the differentially methylated sites identified, and to facilitate the mechanistic investigation of DNA methylation changes in women with PCOS who develop diabetes.

We have presented the first 450K DNA methylation array assessment of women with PCOS compared at initial diagnosis to women with PCOS who later developed diabetes. Despite the limitations of a small sample study, we have identified multiple loci of differential DNA methylation between these clinical phenotypes. We suggest that DNA methylation holds exciting potential for identifying clinically-usable prognostic biomarkers that may help identify women likely to develop diabetes at an early stage where lifestyle interventions may be effective in reducing risks of diabetes and other complications of PCOS.

Declarations

Ethics approval and consent to participate

West Midlands (Black Country) Research Ethics Committee 15/WM/0162

All patients provided written informed consent (copies held in site file by NHS Trust R&D department)

Consent for publication

All authors agree with the manuscript

Availability of data and materials

Array data will be deposited in public repository such as MethDB

Competing interests

None

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Authors' contributions

MK array analyses, Pyrosequencing, data analyses, manuscript preparation

AM Pyrosequencing, manuscript preparation

AAF data analyses, manuscript preparation

PW Patient consent, sample matching, data analyses, manuscript preparation

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References

1. Sirmans, S. M. & Pate, K. A. Epidemiology, diagnosis, and management of polycystic ovary syndrome. *Clin Epidemiol*, **6**, 1–13 https://doi.org/10.2147/CLEP.S37559 (2013).
2. Livadas, S. & Diamanti-Kandarakis, E. Polycystic ovary syndrome: definitions, phenotypes and diagnostic approach. *Front Horm Res*, **40**, 1–21 https://doi.org/10.1159/000341673 (2013).
3. Lakhani, K., Prelevic, G. M., Seifalian, A. M., Atiomo, W. U. & Hardiman, P. Polycystic ovary syndrome, diabetes and cardiovascular disease: risks and risk factors. *J Obstet Gynaecol*, **24** (6), 613–621 https://doi.org/10.1080/01443610400007810 (2004).
4. Moran, L. J., Misso, M. L., Wild, R. A. & Norman, R. J. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update*, **16** (4), 347–363 https://doi.org/10.1093/humupd/dmq001 (2010).
5. Anwar, S. & Shikalgar, N. Prevention of type 2 diabetes mellitus in polycystic ovary syndrome: A review. *Diabetes Metab Syndr Dec*, **11** (Suppl 2), S913–S917 (2017). 10.1016/ j.dsx.2017.07.015
6. Polak, K., Czyzyk, A., Simoncini, T. & Meczekalski, B. New markers of insulin resistance in polycystic ovary syndrome. *J Endocrinol Invest*, **40** (1), 1–8 https://doi.org/10.1007/s40618-016-0523-8 (2017).

7. Jacobsen, V. M. *et al.* Epigenetic association analysis of clinical sub-phenotypes in patients with polycystic ovary syndrome (PCOS). *Gynecol Endocrinol Aug*, **35** (8), 691–694 https://doi.org/10.1080/09513590.2019.1576617 (2019).
8. Toperoff, G. *et al.* Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. *Hum Mol Genet*, **21** (2), 371–383 https://doi.org/10.1093/hmg/ddr472 (2012).
9. Wessely, F. & Emes, R. D. Identification of DNA methylation biomarkers from Infinium arrays. *Front Genet*, **3**, 161 https://doi.org/10.3389/fgene.2012.00161 (2012).
10. Soler-Botija, C., Galvez-Monton, C. & Bayes-Genis, A. Epigenetic Biomarkers in Cardiovascular Diseases. *Front Genet 2019 Oct*, **9**, 10950 https://doi.org/10.3389/fgene.2019.00950 (2019).
11. Chen, Y-A. *et al.* Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. *Epigenetics Feb*, **8** (2), 203–209 https://doi.org/10.4161/epi.23470 (2013).

Figures

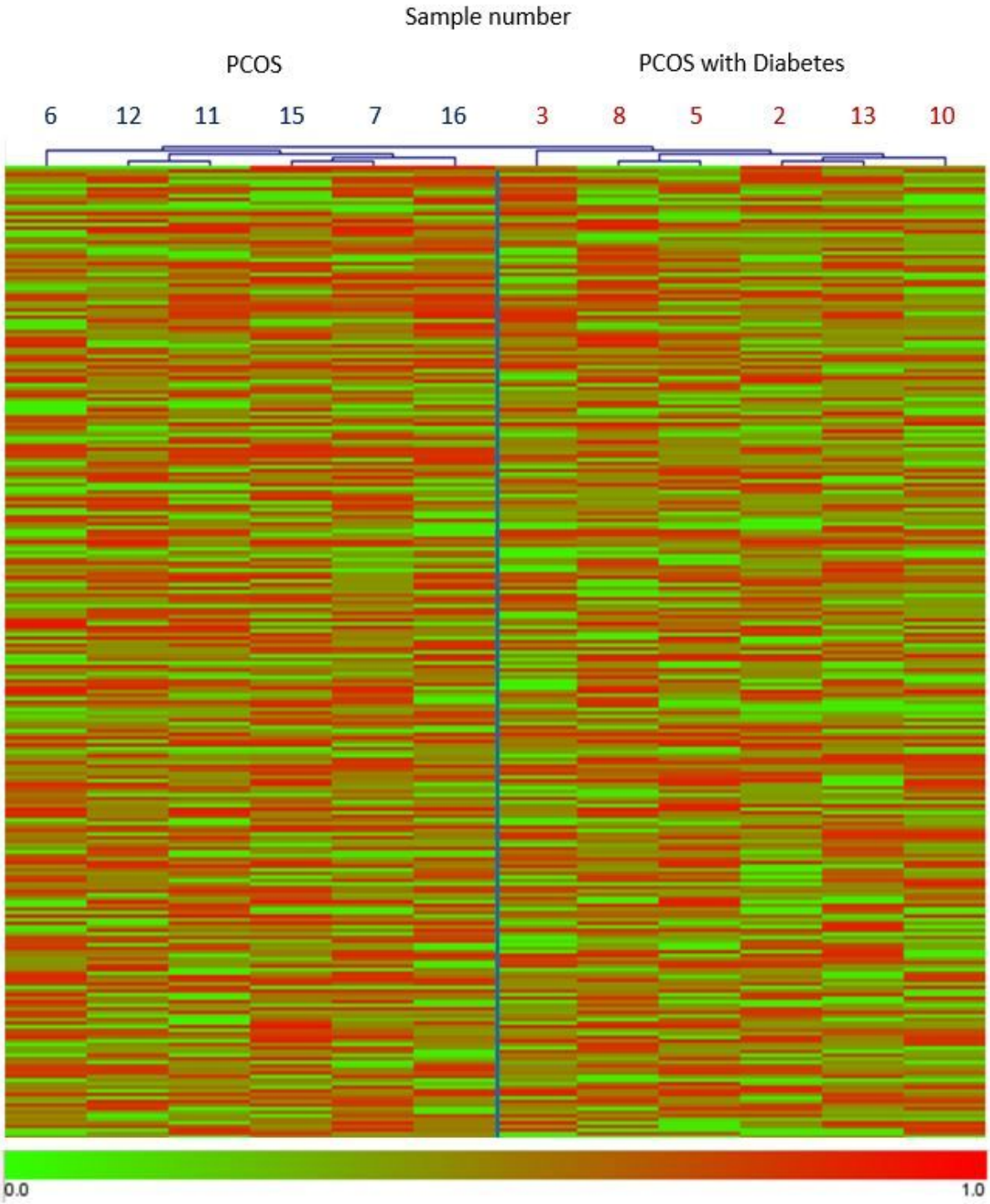

Figure 1

Hierarchical clustering and heatmap of the 273 differentially methylated CpG sites. Heatmap of the differentially methylated CpG sites identified by array analysis between the clinical phenotypes of polycystic ovarian syndrome (control) and polycystic ovarian syndrome with diabetes (cases). The heatmap clusters/separates the six control samples on the left, from the six case samples on the right. Each row represents an individual CpG site, and each column represents an individual sample (listed

above the heatmap). The colour scale beneath represents methylation status: unmethylated is green (β value = 0.0) and fully methylated is red (β value = 1.0).

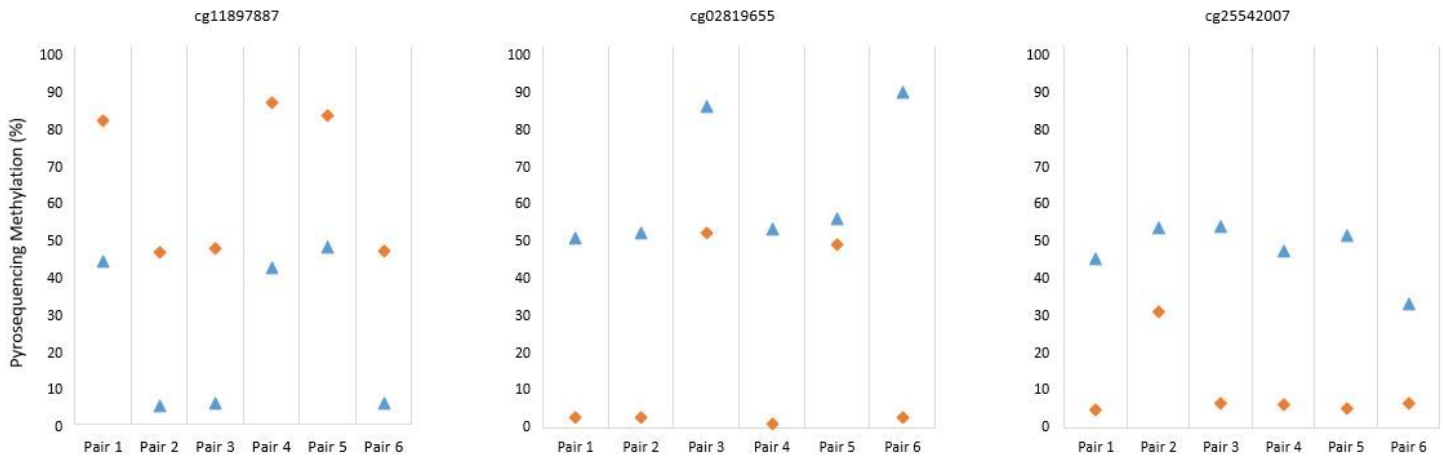

Figure 2

Pyrosequencing of biomarker candidates. Pyrosequencing methylation values (y axis; 0-100%) for all array samples at cg11897887 (left), cg02819655 (middle), and cg25542007 (right), plotted as each matched-pair (x-axis). Each control in the pair is represented by blue triangles, and each case in the pair is represented by an orange diamond. (Pair 1: samples 2 and 16; pair 2: samples 3 and 7; pair 3: samples 5 and 6; pair 4: samples 8 and 15; pair 5: samples 10 and 11; pair 6: samples 13 and 12.)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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