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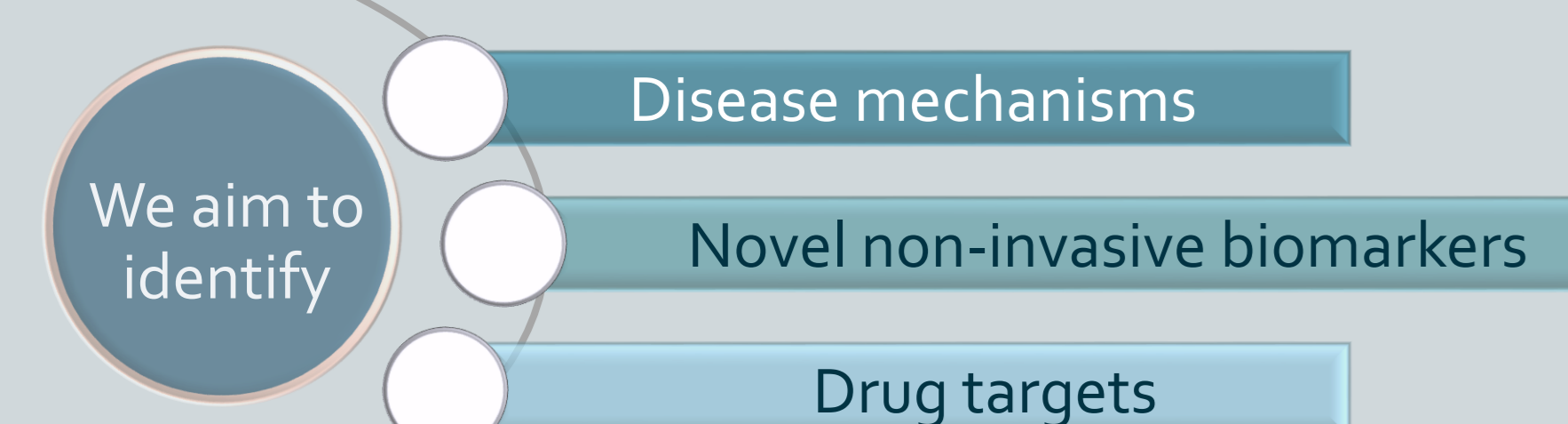
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INTRODUCTION + AIM

PCOS is common and associated with significant comorbidity.

However, its pathogenesis is complex and poorly understood, particularly during adolescence.

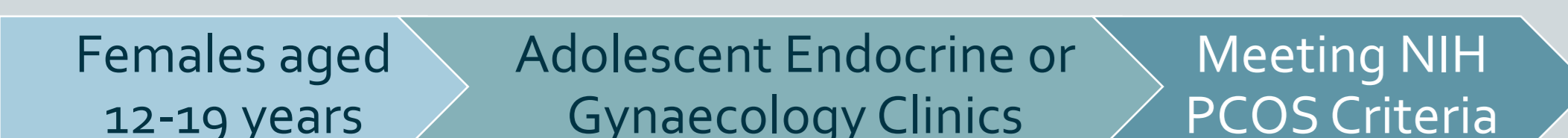
We have developed new methods for deep phenotyping discovery proteomic profiling of urine in PCOS in adolescents.



METHOD

We present the baseline proteomic data from a subset of n=15 samples from our prospective, longitudinal PCOS study (total cohort n=40).

Participant Selection Criteria



We undertook proteomic analysis (nano 2D-LC-QTOF MS^e)

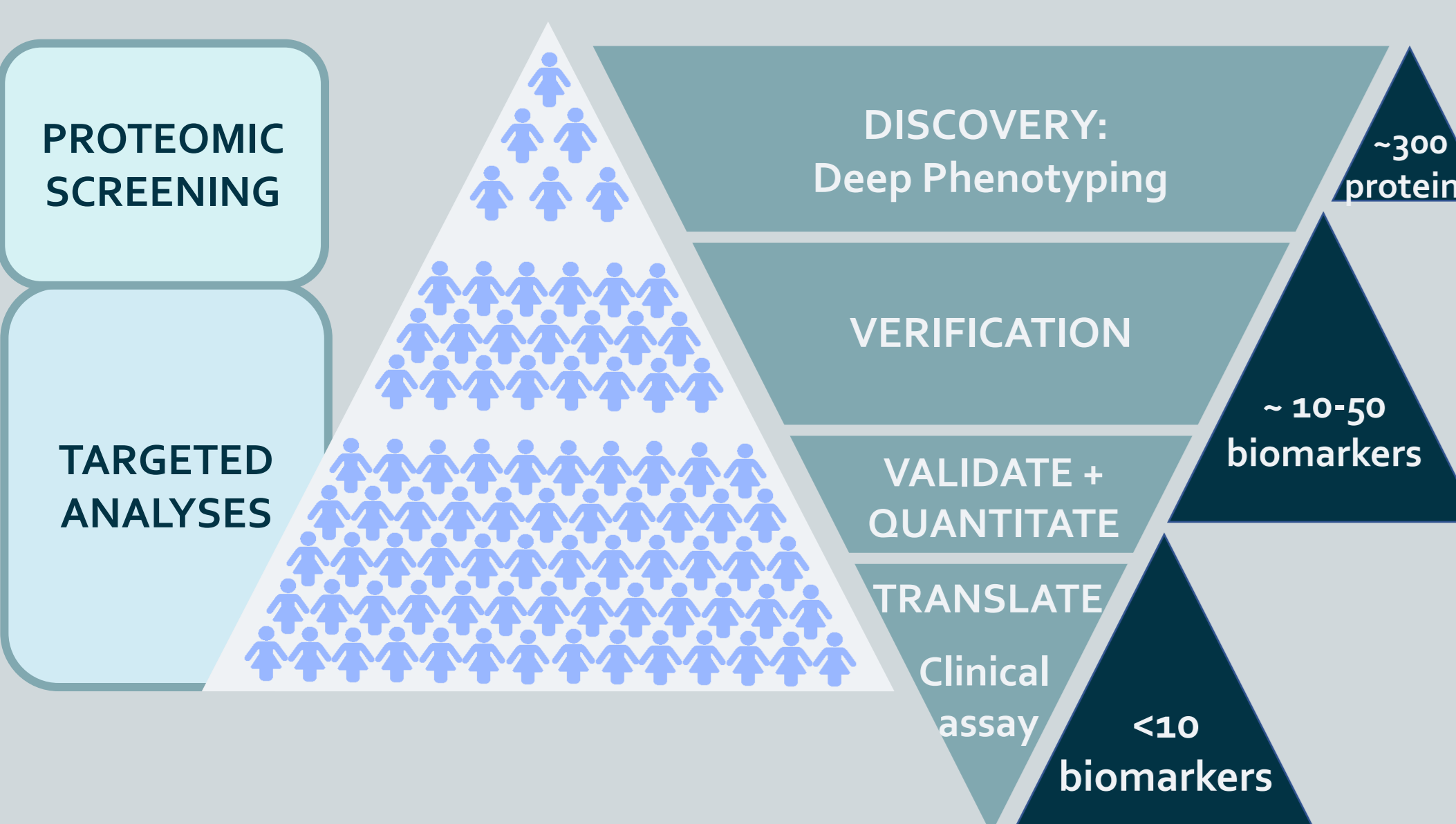
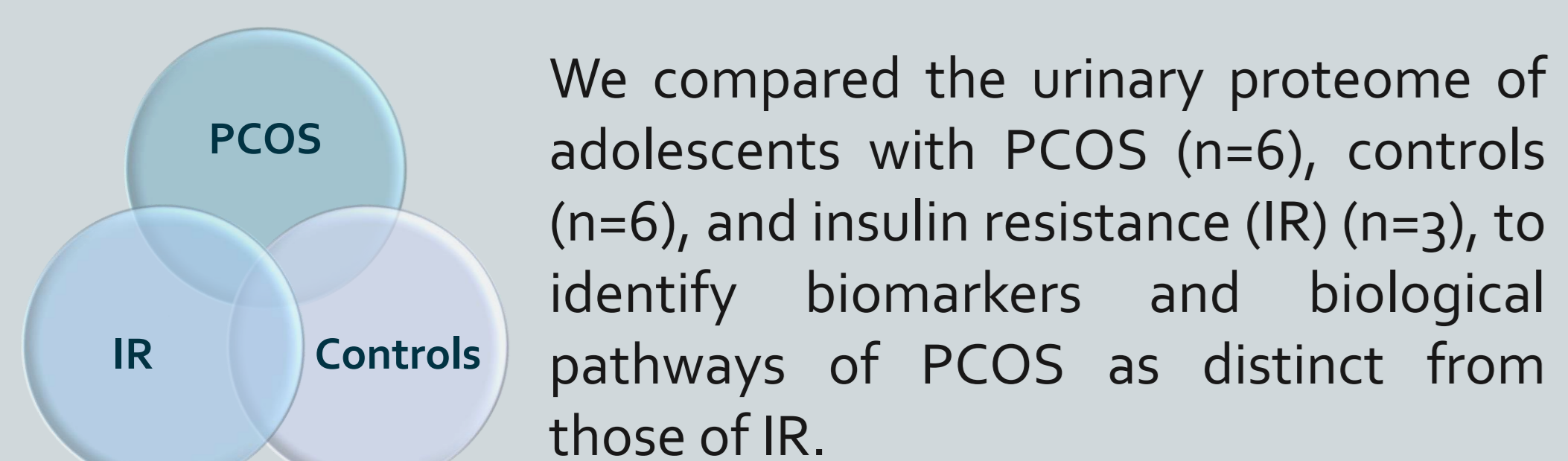


Figure 1 | Schematic displaying process of biomarker identification, from discovery proteomics (undertaken in small cohort, aiming to identify 100's of proteins) to intended clinical assay development (validated in larger cohort, aiming to narrow down to <10 proteins)



RESULTS

Baseline Demographics

- Median **age** 15.0y (range 12.5-18.3y). Mean age at **menarche** 10.9y (SD 1.38)
- Tanner stage** IV (n=17) + V (n=23)

Protein Identification

- We identified **3,793 proteins** across the PCOS, IR and control cohorts.
- n=314 were significantly and differentially expressed proteins (DEPs) in the PCOS cohort vs. the control cohort. n=397 were DEPS in PCOS vs. the IR cohort.
- n=66** DEPs were identified in both cohorts i.e. **consensus proteins** significantly different in the PCOS cohort in comparison to both controls and IR. These 66 consensus DEPs are **potential biomarkers** for PCOS.

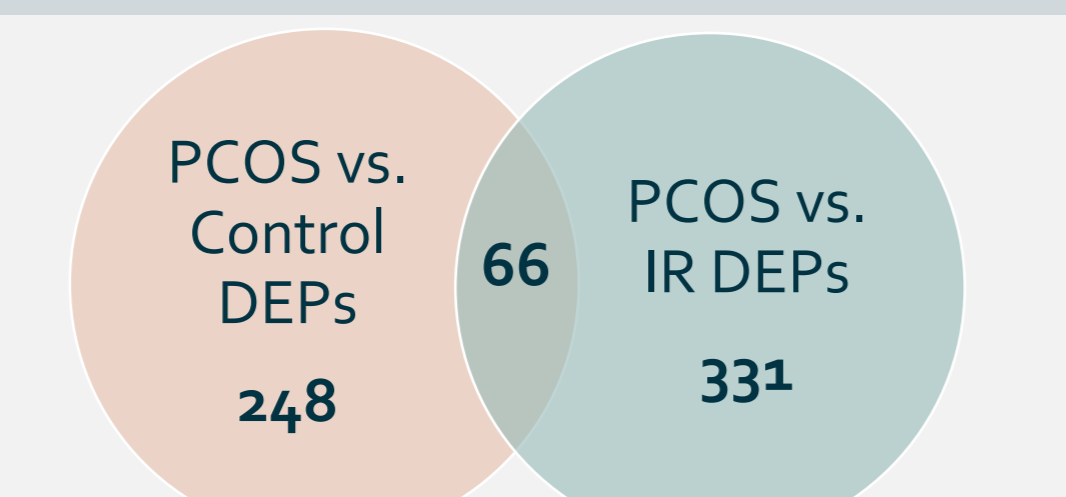


Figure 2 | Venn Diagram of DEPs
Of 645 individual DEPs across cohorts, 248 were identified in the PCOS vs. control group only, 331 were identified in the PCOS vs. IR group only and 66 were identified in both groups.

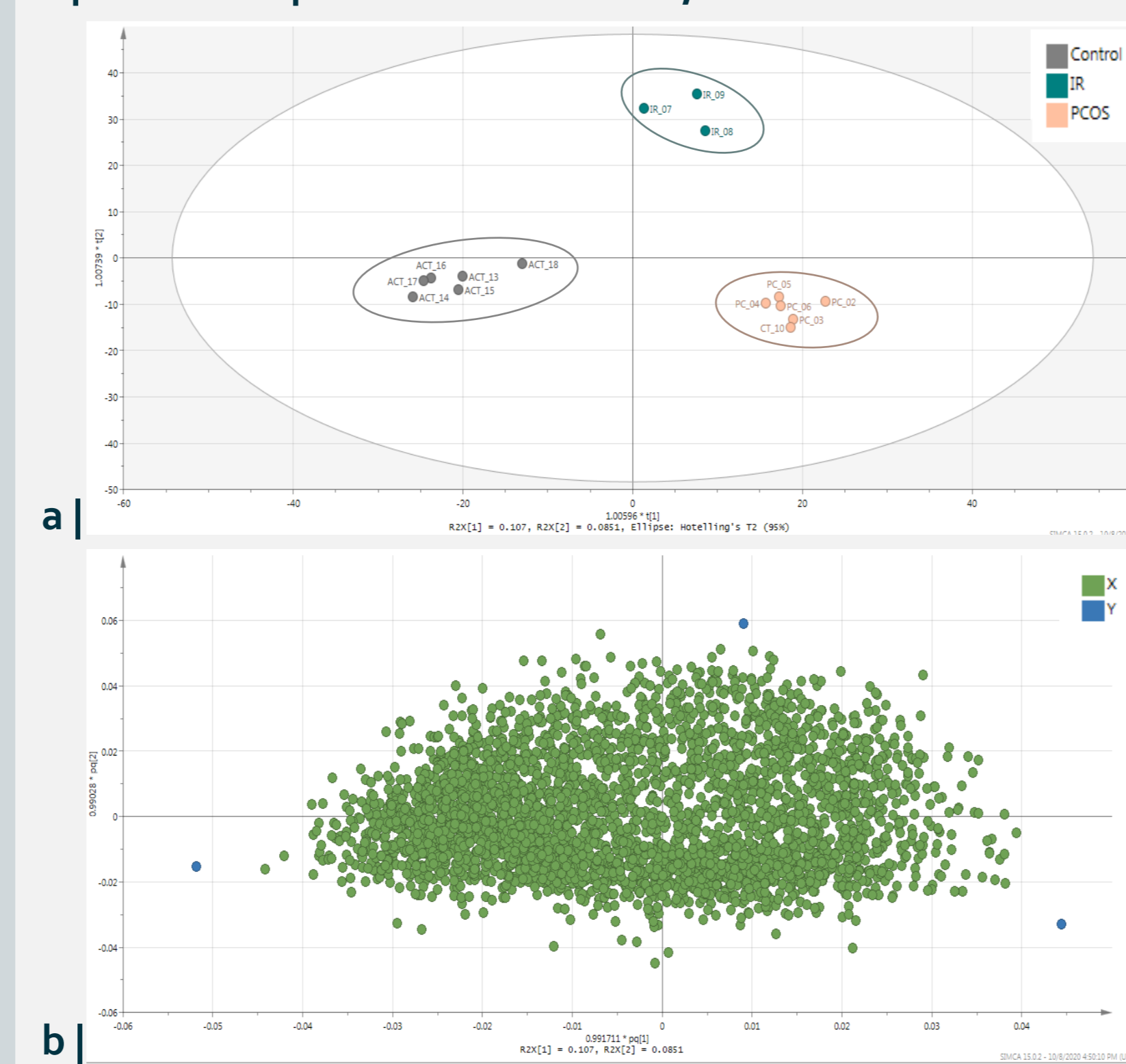
- Consensus DEPs had a median fold-change of -2.2 (range -16.6 – +1775.5) and eight were upregulated in the PCOS cohort.

SIMCA Multivariate Analysis

We performed multivariate *SIMCA*[®] analyses on all proteins to identify cohort-level differences and similarities in the proteomes.

OPLS-DA analysis showed distinct clustering within, and separation between all cohorts (fig. 3), indicating **quantifiable differences between the proteome** of all three cohorts (PCOS vs. control vs. IR), but **similarities within them**.

Figures 3a + 3b | SIMCA Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) plots for all proteins in the PCOS, control and IR cohorts.



a | OPLS-DA score scatter plot. Participants are denoted by a peach (PCOS, n=6), grey (control, n=6) or turquoise (IR, n=3) data point. Observations in close proximity to each other will have similar properties.

b | OPLS-DA loading scatter plot. When fig. 3b is overlain onto fig. 3a, the individual proteins which are most responsible for variations in the observations can be identified.

X variables (green) represent individual proteins and the Y response variables (blue) represent the PCOS (right), control (left), and IR (top) cohorts. Proteins (X variables) which are closer to Y variables, represent those with greater abundance in that cohort.

Gene Ontology Analysis

- Gene ontology (GO) is the study of the function of genes and their proteins. We undertook GO analysis of all consensus DEPs to better understand their role in PCOS and wider biological processes.
- These 66 consensus proteins are involved in the processes shown below in **figure 4**. A significant number of these proteins were identified as mediators of **immune and inflammatory pathways**.

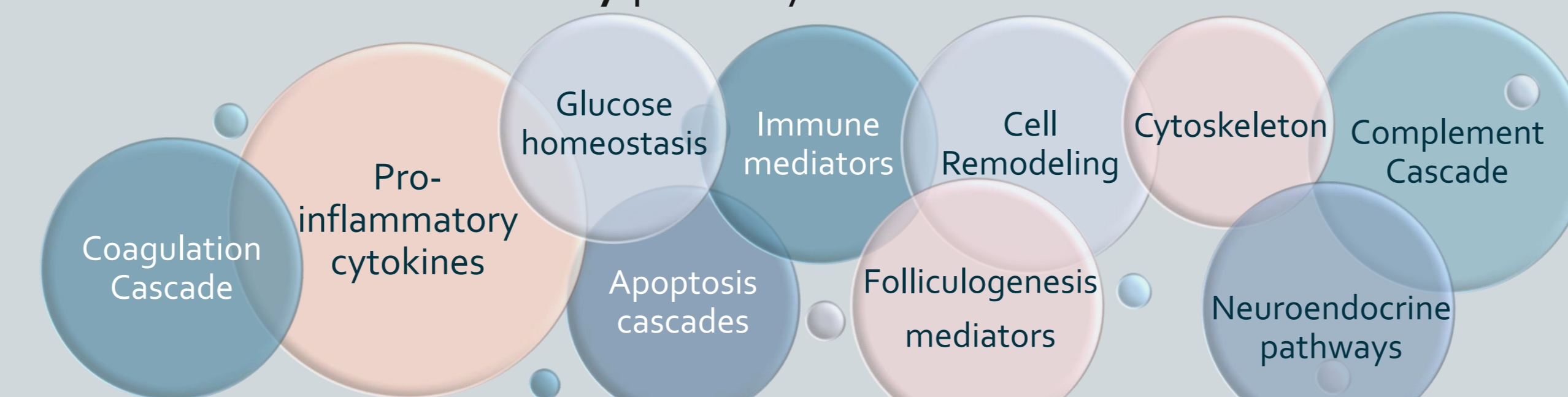


Figure 4 | The main functions of PCOS consensus differentially expressed proteins

CONCLUSIONS

This novel study has utilised **non-invasive matrices to map the proteome of PCOS** in adolescents.

We have developed **highly sensitive proteomic analysis techniques** and **identified thousands of proteins** in urine and identified **66 potential novel biomarkers**.

We have provided promising insight into the **molecular pathways of PCOS**, and demonstrated that **inflammation** could be a major contributory factor in its pathophysiology.

To confirm these findings, we are performing targeted multiplexed assays comprising 70 inflammatory proteins on our entire longitudinal PCOS cohort.

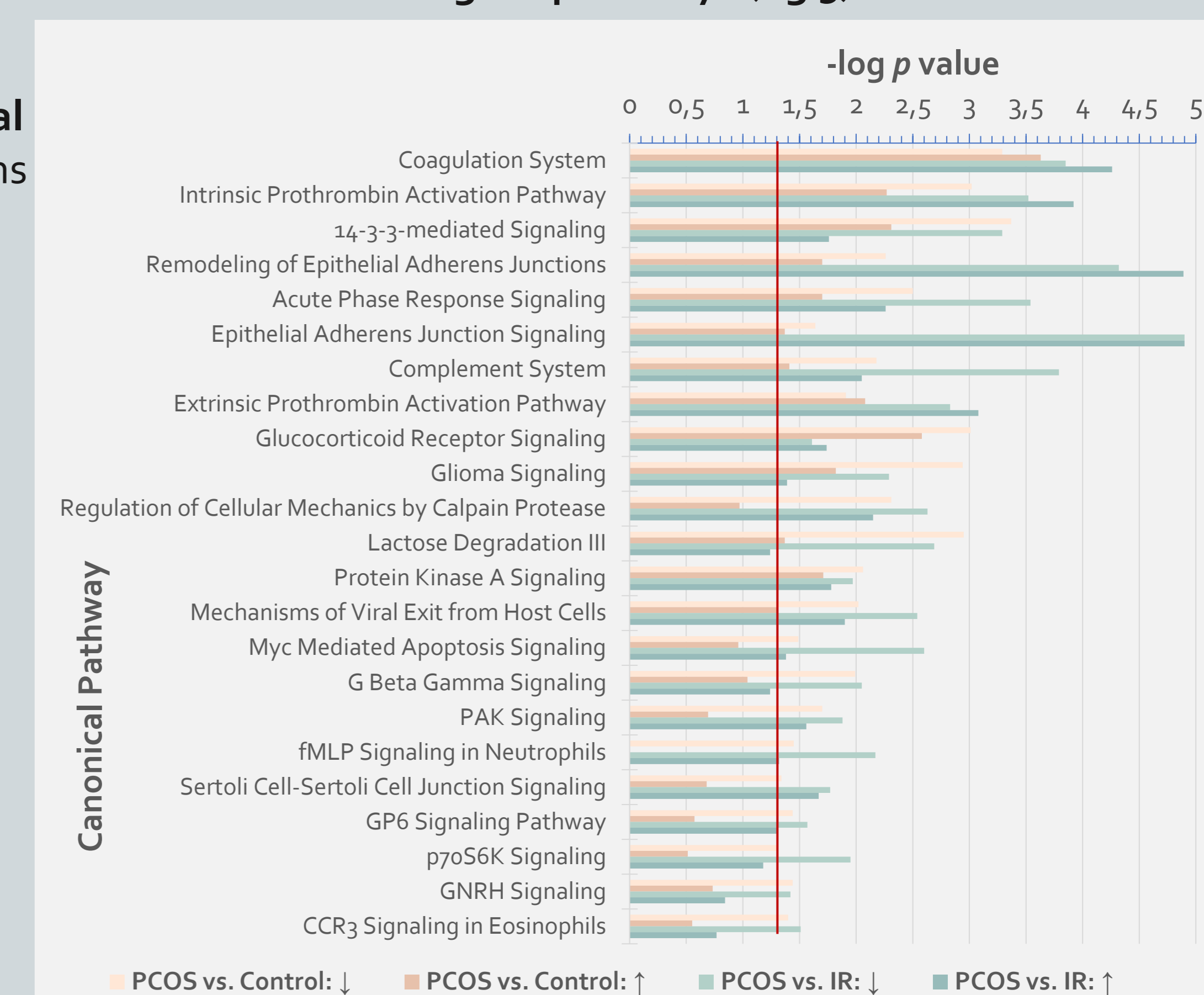
Bioinformatic Ingenuity Pathway Analysis

- Significant proteins were imported into *Ingenuity*[®] to identify biological pathways associated with PCOS in comparison to controls/IR. We identified **23 significant 'canonical' biological pathways** (fig 5).

- 43% (10/23) of all significant pathways were associated with **inflammatory/immunological responses** and **thrombotic/fibrinolytic systems**
- 22% (5/23) related to **glucose homeostasis/insulin resistance** and **hyperandrogenism**
- 35% (8/23) related to **folliculogenesis, remodelling, the cytoskeleton,, apoptosis + autophagy**

Figure 5 | Consensus Canonical Pathways

Comparison of canonical pathways in PCOS vs. control/IR cohorts in both "high stringency sets" (confidence value ≥ 20 , unique peptides ≥ 2) and "low stringency sets" (confidence ≥ 15 , unique peptides ≥ 1). Significance expressed as $-\log p$ value. Non-axial red line denotes significance ($-\log p > 1.3$ equivalent to $p < 0.05$). Pathways displayed in order of average ranking of $-\log p$ across all stringency sets.



Diseases and Biological Function Analysis

We also utilised *Ingenuity*[®] to identify how these 66 consensus proteins and potential biomarkers correlate with biological functions and diseases (figure 6). The most significant process associated with PCOS was the **inflammatory response**.

Additionally, the majority of these abundant processes and diseases are linked to the inflammatory response.

This, in addition to the evidence from our pathway and gene ontology analysis highlights the significance of inflammatory mechanisms in the pathophysiology of PCOS and adds to this body of evidence.

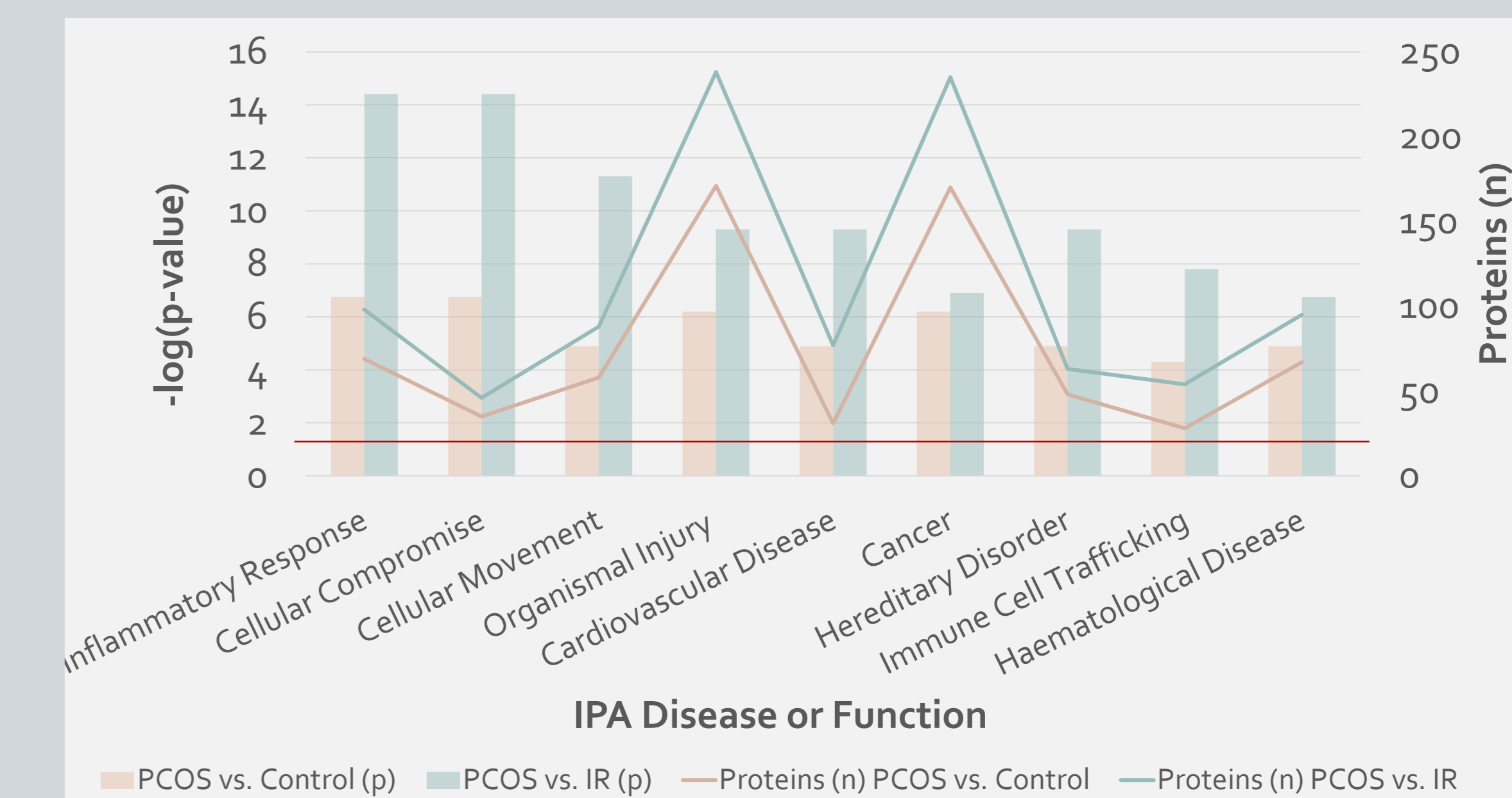


Figure 6 | Consensus IPA Diseases and Functions
Each pair of bars represents a disease/function, listed in order of significance. Trendlines indicate the number of proteins in our dataset associated with a disease/function. The red non-axial line denotes significance ($-\log > 1.3 = p < 0.05$).

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