Pre-eclampsia risk stratification for low risk 1st pregnancies: First results of a new LC-MS based multiplex metabolite assay.

Liz Bond[1,5], Grégoire Thomas[2], Caroline Nolan[1,5], Philip Baker[3,5,6], Louise Kenny[4,5,6], Robin Tuytten[1,5]

[1]Metabolomic Diagnostics, Little Island, Cork, Ireland; [2]Squ4Re, Lokeren, Belgium; [3]Gravida: National Centre for Growth and Development, [3]The University of Auckland, New Zealand; [4]The Irish Centre for Fetal and Neonatal Translational Research, Cork University Maternity Hospital, Cork, Ireland; [5] on behalf of the IMproved Pregnancy Outcomes by Early Detection consortium (IMPROvED), [6]on behalf of Screening fOr Pregnancy Outcomes consortium (SCOPE)

INTRODUCTION

- With pre-eclampsia affecting 2-8% of all pregnancies, screening for preeclampsia is a focus of prenatal care.
- Current screening protocols are largely based on clinical risk factors, however these fail to accurately predict pre-eclampsia risk in 1st time pregnant women.
- An unbiased metabolomics biomarker discovery effort revealed that combinations of blood-borne metabolite biomarkers have the potential to predict pre-eclampsia at about 15 weeks of gestation.[1](Kenny et al, 2010). These findings founded the base for a company focusing on the development of a metabolites based pre-eclampsia (PE) risk prediction test.
- A prototype multiplex assay for the analysis of 40 metabolite biomarkers was developed using liquid chromatography-mass spectrometry (LC-MS).

OBJECTIVES

- Technical fit-for-purpose testing of the prototype LC-MS pipeline.
- Independent verification of the risk stratification potential of the biomarkers through a case control study.
- Development of multivariate prediction that identify within a population of low risk 1st time pregnant women,
  1. a group of pregnancies which has an increased risk of preeclampsia and/or
  2. a group of pregnancies who has a decreased risk of preeclampsia.

Enrichment targets were derived from the pre-eclampsia risk in primiparous women as shown in Figure 1.

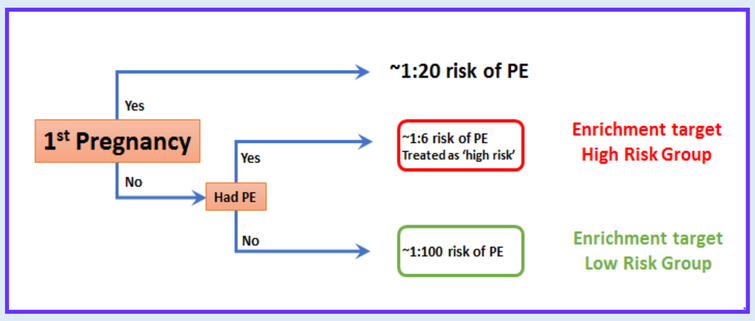


Figure 1: *Enrichment targets for the metabolite based multivariate models. The prior risks are based on Hernández-Diáz et al. BMJ 2009; 338:b2255. & Kenny et al, Hypertension 2014; 64:644-652.*

METHODS

ANALYTICAL PIPELINE CONSTITUENTS

- Dedicated EDTA plasma work-up for the extraction of metabolites, with the following key attributes:
  1. Sample fortification with a mixture of Stable Isotope Labelled (SIL) metabolites prior to work-up a) to compensate for experimental variability and b) to relatively quantify and thus compare metabolite levels across all samples.
  2. Throughput of 96 blood samples per day .
- Two dedicated LC-MS assays, with the following key attributes
  1. Multiplex analyses of 40 metabolites and 26 SIL standards distributed over 2 LC-MS assays
  2. LC: Agilent 1260 HPLC set-up, Reversed Phase LC (Agilent Pursuit PFP), 10 min gradient run
  3. MS: Agilent 6460 Triple quadrupole MS equipped with Agilent Jet Stream source, 2 multiple reaction monitoring transitions per target compound
- Quality assurance protocols

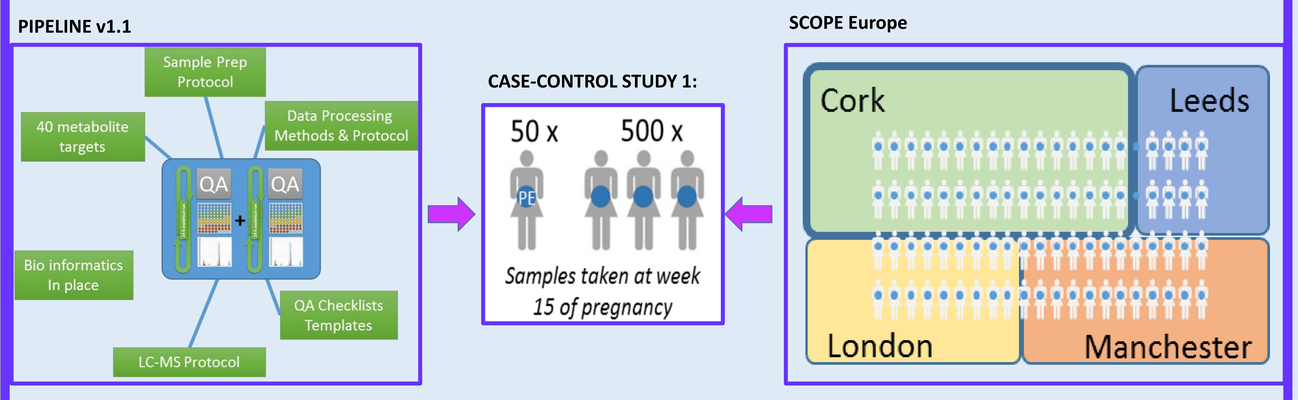


Figure 2: *Schematic overview of the study performed. A dedicated LC-MS pipeline targeting 40 metabolite biomarkers was applied to a 50:500 case:control study defined within the European arm of the Screening for Pregnancy Endpoints (SCOPE) study*

CASE CONTROL STUDY - DESIGN

- CCS_1 was nested within the European arm of the SCOPE study (n=2456, PE = 100, non-PE = 2356)
- Samples from Cork cohort only
- 1:10 Case:Control ratio; 50 cases vs. 500 Controls
- Cases and controls* randomly selected (*non-PE, other comorbidities present)

CASE CONTROL STUDY - TECHNICAL

- 15% replicates were considered
- All samples were randomized and lab personnel blinded to outcome
- Since the platform was duplicated (2 LC-MS assays), all samples were processed twice and were analysed 2 times by LC-MS. Each of the 2 sub-studies was independently randomized
- Inclusive QC samples 1500 + analyses were performed.

RESULTS

ASSESSMENT ANALTYICAL PIPELINE

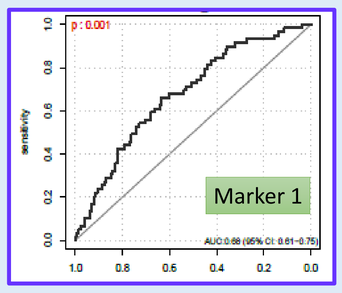
- For 9/40 targets there was more than 20% missing data within CCS_1, the data of these assays was discarded.
- Precision for the remaining 31 assays was satisfactory with 71% of the assays having a %CV ≤ 15*.
  (*Precision metrics are determined for the whole analytical pipeline: sample work-up, LC-MS analysis, and data processing).
- The use of SIL standards was confirmed to compensate for most of the technical variability.

class	Assay	Dedicated ISTD	%CV	missing values
Keto Fatty Acids	Ro1.met_035	yes-(3H)7		100%
Amino Acids	Ro2.met_025	No		98%
Steroids	Ro2.met_030	No		98%
Amino Acids	Ro2.met_042	yes-(13C)2		93%
Fatty Acids	Ro2.met_015	No		91%
Amino Acids	Ro2.met_041	yes-(3H)6		88%
Vitamin D3 - derivatives	Ro1.met_014	yes - (3H)3		84%
Keto Fatty Acids	Ro1.met_008	no		60%
Dicarboxylic Acids	Ro1.met_006	no		49%
phospholipids	Ro1.met_039	yes-(3H)7	22%	11%
Amino Acids	Ro2.met_032	no	34%	9%
Vitamin D3 - derivatives	Ro1.met_002	yes - (3H)3	18%	5%
Vitamin D3 - derivatives	Ro1.met_022	yes-(3H)3	35%	4%
Dicarboxylic Acids	Ro1.met_004_009	yes - (3H)8	18%	1%
Ketone	Ro1.met_023	yes-(3H)6	4%	0
Amino Acids	Ro1.met_007	yes - (3H)3	5%	0
Amino Acids	Ro2.met_031	yes-(13C)5	6%	0
Amino Acids	Ro2.met_028	yes-(13C)6	7%	0
Amino Acids	Ro2.met_038	yes-(3H)6	8%	0
Keto Fatty Acids	Ro2.met_005	yes-(3H)4	8%	0
Sugars	Ro1.met_024	yes-(13C)6	9%	0
Keto Fatty acids	Ro1.met_033	no	9%	0
Keto Fatty Acids	Ro2.met_003	no	10%	0
Fatty Acids	Ro2.met_011	yes-(3H)8	10%	0
Fatty Acids	Ro1.met_020	yes-(3H)5	10%	0
Amino Acids	Ro2.met_010	yes-(13C)3	11%	0
Ketone	Ro1.met_036	no	11%	0
Fatty Acids	Ro2.met_027	yes-(3H)4	12%	0
Steroids	Ro2.met_016	No	13%	0
Amino Acids	Ro2.met_026	No	13%	0
Fatty Acids	Ro1.met_034	yes-(13C)5	13%	0
Carnitines	Ro1.met_018	yes - (3H)3	13%	0
Amino Acids	Ro2.met_017	yes-(3H)7	14%	0
Carnitines	Ro2.met_019	yes - (3H)3	15%	0
Carnitines	Ro2.met_037	yes - (3H)3	15%	0
Amino Acids	Ro2.met_012	yes-(13C)6	15%	0
Lipid	Ro1.met_021	yes-(3H)5	16%	0
phospholipids	Ro1.met_040	No	17%	0
Amino Acids	Ro2.met_013	No	19%	0
Fatty Acids	Ro2.met_029	No	23%	0

Table 1: *Summary of the 40 metabolites assayed in case-control study: Metabolite class; assayed in 1st (Ro1) or 2nd (Ro2) LC-MS analysis; Dedicated SIL standard; Precision based on 15% replicate analyses; and % missing values.*

ASSESSMENT SINGLE BIOMARKER POTENTIAL

- The predictive performance of single biomarkers was assessed by means of the Receiver Operating Characteristic statistic (ROC). Metabolites with an area under the curve (AUC) significantly different from the null hypothesis (AUC = 0.5) are reported (lower limit of the 95% Confidence Interval of the ROC-AUC ≥0.5)
- 7/31 metabolites assayed had significant predictive power for pre-eclampsia (Table 2)



→ ***Table 2:*** *Single marker prediction metrics F*
← ***Figure 3:*** *ROC curve for the metabolite with the strongest stand-alone predictive performance*

Marker	AUC	95% CI	Missingness	trend	%CV
Marker 1	0.68	[0.61 – 0.76]	-	PE > Ctrls	16%
Marker 2	0.61	[0.53 – 0.69]	-	PE > Ctrls	9%
Marker 3	0.60	[0.52 – 0.68]	-	PE > Ctrls	13%
Marker 4	0.59	[0.51 – 0.68]	-	PE < Ctrls	11%
Marker 5	0.59	[0.51 – 0.67]	-	PE > Ctrls	15%
Marker 6	0.58	[0.51 – 0.67]	PE:1; Ctrl:19	PE < Ctrls	18%
Marker 7	0.58	[0.50 – 0.65]	PE:3; Ctrl:20	PE > Ctrls	35%

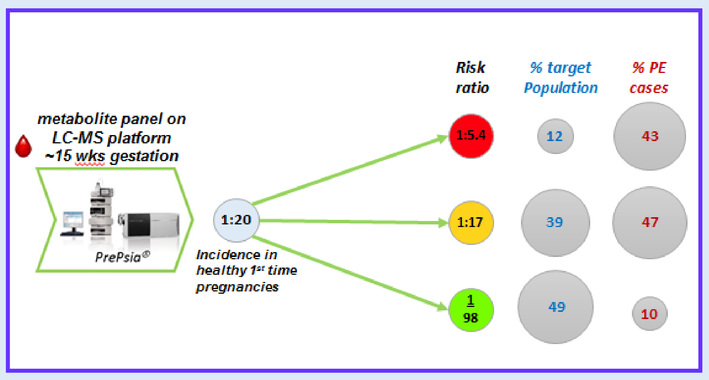
MULTIVARIATE ANALYSES

- Application of logistic regression to develop predictive models for PE.
- Models with a maximum of up to 5 variables (metabolites and selected clinical parameters)
- Models selected based on discriminative performance, significance of the variables
- Identify models which fulfil pre-set enrichment targets

Figure 2: *Schematic overview of the data analysis pipeline.*

RESULTS MULTIVARIATE ANALYSES

- Multimarker models were found which identify a group at increased risk of pre-eclampsia within a population of nulliparous women without classic clinical risk factors. Post-test probability is ≥ 1 in 6.
- Multimarker models were found which identify a group at decreased risk of pre-eclampsia within a population of nulliparous women without classic clinical risk factors. Post-test probability is ~ 1 in 100.

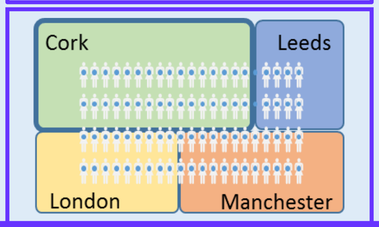


CONCLUSIONS

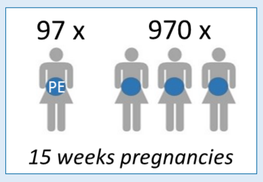
- LC-MS was confirmed as a viable platform for multiplex analysis of metabolite biomarkers relevant to the prediction of pre-eclampsia.
- Combining the levels of multiple metabolite markers in multivariate models enables stratification of otherwise low risk nulliparous pregnant women into high risk or low risk groups for pre-eclampsia at about 15 weeks of gestation.

NEXT STEPS

ASSAYS
- Improved robustness
- Single multiplex LC-MS analysis



CASE-CONTROL STUDY 2:



- Robust prediction models

Clinical Validation of the Metabolite based test in large scale European , multicentre phase IIa clinical study IMPROvED (Navaratnam et al. 2013)[2].

IMPROvED: **Im**proved **P**regnancy **O**utcomes by **E**arly **D**etection

PrePsia V1.0

[1] Kenny, L. C. *et al. Hypertension* **56,** 741-9 (2010); [2] North, R. A. *et al.* t. *BMJ* **342,** d1875 (2011); [3] Navaratnam, K. *et al. BMC Pregnancy Childbirth* **13,** 226 (2013)

The authors gratefully acknowledge funding from the EU-HEALTH Project IMPROvED (305169) of the Seventh Framework Programme (FP7).

The main goal of IMPROvED is to develop a clinically robust predictive blood test for pre-eclampsia, using innovative technologies and utilising novel metabolite and protein biomarkers. www.fp7-improved.eu

The authors also gratefully acknowledge
1) The SCOPE consortium for the collaboration opportunity
2) instrument and technical support from Agilent Technologies.

www.metabolomicdiagnostics.com