METABOLOMICS AS NEW APPROACH FOR INVESTIGATING EXPOSURE TO ENVIRONMENTAL CHEMICALS AND ASSOCIATED ENDOCRINE DISRUPTION SITUATIONS: HOPE OR HYPE?

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Introduction

Although not that particularly recent, the so-called 'omic techniques (transcriptomic, proteomic, metabolomic) are gaining a huge interest with substantial developments and applications in various research areas over the last few years. The fundamental idea is to explore the "life complexity" using unrestrictive descriptive methodologies[1,2]. Thus, these approaches are all based on the generation of large sets of descriptors expected to characterise the biological system under investigation. These descriptors refer to the genomic, proteomic or metabolic species. With regards to the metabolome, the monitored signals correspond to chemical substances (so-called metabolites) accessible to the analysis, which are final products formed after the complex transcription, transduction, and regulation mechanisms. It represents a comprehensive dataset that defines the small molecules present in a cell, tissue, all organism or biological fluid. The general principle of metabolomics is then to characterise biological samples by the production of a chemical profile so-called metabolic fingerprint. When performed on large sample sets, the comparison of such multi-endpoint measurements is expected to reveal potential similarities or differences between the analysed samples. From the information contained within the dataset, it is theoretically possible to establish relationships between metabolite levels and cellular response to chemical and nutritional stimuli as well as disease state and other stressors. Thus the interrogation of the metabolome may present a new way of investigating the complex area of chemical environment contamination[3]. From an analytical point of view, the most widely used technique for metabolomics has been nuclear magnetic resonance (NMR). However, mass spectrometry (MS) is becoming more and more widely used in this field.

LABERCA has been developing for several years global characterization approaches (notably metabolomics) of biological fluids and / or food products to characterize the impact of chemical contaminants at various scales, with the ambition to ultimately highlight biomarkers of exposure and / or effect. This has given rise to a number of methodological developments[4-9] leading to the definition and validation of an integrated analytical strategy that today represents the laboratory's pipeline with regard to this type of studies[10,11].

In this general context, the aim of this study was to characterize a specific endocrine disruption situation related to human testicular function. Indeed, an increased prevalence of disorders affecting the reproductive function (cryptorchidism, hypospadias, low sperm concentration, testis germ cell cancer) has been noticed during the past decades. These disorders are thought to be different symptoms of the same entity: the testicular dysgenesis syndrome (TDS)[12], linked to disruption of testis development during foetal life. Pieces of evidence suggest that common environmental chemicals probably acting together or in combination with other factors (genetic, lifestyle) could contribute to TDS. However, there are numerous obstacles to proving this scientifically, including major analytical challenges. In the frame of a 7th FP EU project (DEER), a metabolomic approach was developped to assess its potential for characterizing a specific endocrine disruption situation related to reduction in spermatozoa concentration. To this end, metabolic fingerprinting on serum samples collected from men presenting different semen qualities were performed in order to characterize patterns of metabolites associated to a reduction in spermatozoa concentration. Ultimately, the potential link between the generated metabolomic fingerprints, and the exposition of the individual to chemicals will be investigated.

Materials and methods

Serum samples (97) were collected at Rigshospitalet (Copenhagen) from Danish young men presenting different semen qualities (spermatozoa concentrations, Table 1). Serum was separated and kept frozen at

minus 20 °C until analysis. Serum samples (100 µL) were filtered on PALL centrifugal devices (NANOSEP OMEGA, cut of at 10 kDa) at 10 000 rpm for 30 min in order to remove high molecular weight proteins. 60 µL of the filtrate were mixed with 20 µL of an internal standard solution. Metabolomic fingerprinting was performed on a Agilent 1200 HPLC system including an autosampler, a binary pump and coupled to a Finnigan LTQ-Orbitrap™ hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). All solvents and reagents were of analytical or HPLC grade quality and purchased from Solvent Documentation Synthesis (SDS, Peypin, France). Chromatographic separation was performed on an Hypersyl-Gold column (100 mm x 2.1 mm x 1.9 µm particle size, Thermo Fisher Scientific). Mobile phase consisted in water containing 0.1 % acetic acid (A) and acetonitrile containing 0.1 % acetic acid (B). The used elution gradient (A:B, v/v) was as follow: 95:5 from 0 to 2.4 min; 75:25 at 4.5 min; 30:70 at 11 min; and 0:100 at 14 min. The injected volume was 10 µL, the flow rate was 0.4 mL/min and the temperature of the column was maintained at 35°C. The HPLC column was connected without splitting to the electrospray interface operating in positive or negative mode. Spray voltage was set to 5 kV or 4 kV, respectively. The temperature of the heated transfer capillary was maintained at 350°C. Sheath and auxiliary nitrogen gas were applied to help the evaporation of the solvent at a flow rate of 55 and 6 arbitrary units respectively. Full scan mass spectra were acquired from m/z 50 to 800 at a resolution of R=30,000 (FWHM) in centroid mode. Data processing was performed according to the open-source XCMS software. XCMS parameters for the R language were implemented in an automation script. The interval of m/z value for peak picking was set to 0.1, the signal to noise ratio threshold was set to 10, the group band-width was set to 30 and the minimum fraction was set to 0.8. Finally, identified ions in the samples are sorted out to generate a two-dimensional data table, in which rows and columns represent the samples and the peak areas of metabolites, respectively. Multivariate statistics were performed on this table using SIMCA-P+® software (Version 12, Umetrics AB, Sweden). Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was used for building up predictive models and the quality of the models was evaluated by three parameters: $R^2(X)$, corresponding to the proportion of the total variance of the dependant variables that is explained by the model, $R^2(Y)$, defining the proportion of the total variance of the response variable (i.e. the class of the samples) explained by the model, and $Q^2(Y)$, which is similar to $R^2(Y)$ excepted that it is computed by cross-validation.

Table 1: Descriptive statistics (number, mean, maximum and minimum spermatozoa concentrations) related to serum samples collected from healthy men presenting different semen qualities.

	Low sperm conc.	Intermediate sperm conc.	High sperm conc.
n	33	31	33
Mean Spermatozoa Conc. (10^6/mL)	11 ± 6	58 ± 9	167± 67
Min (10^6/mL)	0.3	45	100
Max (10^6/mL)	19	72	392

Results

The sera metabolomic fingerprints acquired in positive and negative ionisation mode from men presenting a poor or a high semen quality were both subjected to an OPLS-DA analysis. The two classes of patients were well separated considering this multivariate model based on the whole fingerprint (961 ions detected in positive mode and 477 in negative mode). The performance characteristics of this multivariate model from a descriptive and predictive point of view were as follows: $R^2(X)=0.738$, $R^2(Y)=0.948$ and $Q^2(Y)=0.778$. The values of $R^2(Y)$ and $Q^2(Y)$ indicate that most of the variance related to the response variable (i.e. sample class) is explained by the OPLS-DA model and that the model has a good predictive ability. This was further confirmed through external predictions: 1/3 of the data was attempted to be independently predicted by the model. None of these samples appeared misclassified. This result demonstrated that sera metabolic differences exist between men presenting a poor versus a good semen quality (Figure 1).

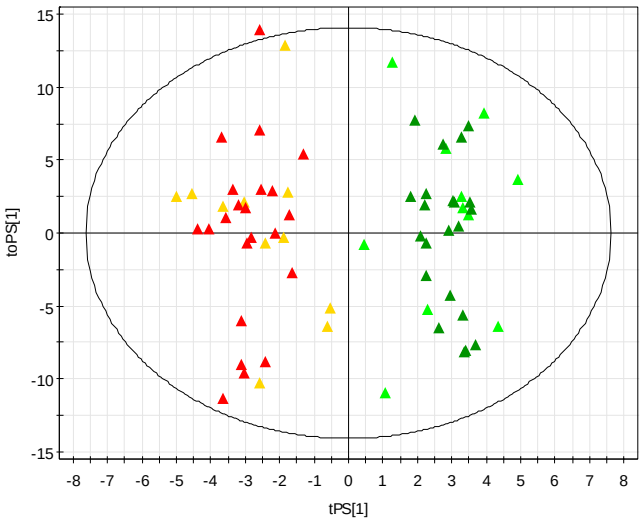

Figure 1: T-predicted score plot (OPLS-DA) observed for 66 metabolomic fingerprints generated from serum after negative electrospray ionisation. The red / yellow and green / dark green triangles stand for patients presenting a poor and high semen quality, respectively. Observations that allowed to construct the model are in red and dark green. Observations that have been predicted by the model are in yellow and green

In a second step, a model no longer based on the class membership of the samples, i.e. poor or high sperm concentration, but on the really measured spermatozoa concentrations values associated to these samples was attempted to be built (Figure 2a).

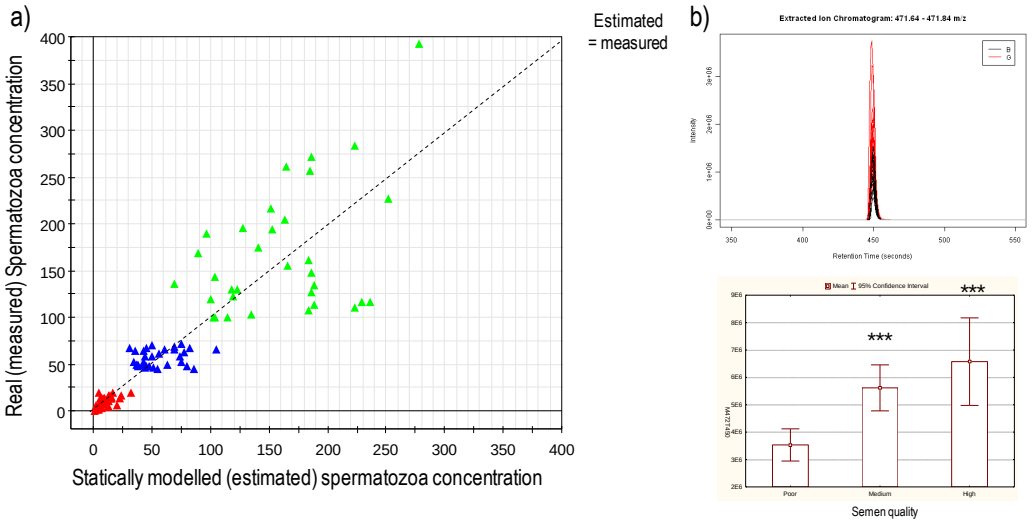

Figure 2: a) observed versus predicted plot observed for 97 sera metabolomic fingerprints collected in negative mode on patients presenting poor (red triangles), medium (blue triangles) and high (green triangles) semen qualities on the basis of 477 variables, b) examples of overlaid extracted ion chromatograms corresponding to potential metabolites of interest detected in serum samples. Chromatograms in red correspond to men presenting a high semen quality and chromatograms in black correspond to men presenting a poor semen quality. Histograms represent the mean and the 95% confidence interval of each group.

The characteristics of this quantitative model were: $R^2(X)=0.501$, $R^2(Y)=0.883$ and $Q^2(Y)=0.659$. Most of the variance related to the spermatozoa concentration (quantitative Y-variable) appears explained by the model. A good correlation can be observed between the real spermatozoa concentration and the statistically estimated

ones, thus confirming that the metabolic differences highlighted between men presenting poor and high sperm quality are directly linked to this biological parameter. Several metabolites were pointed out as correlated to the spermatozoa concentration and needs now to be structurally investigated (Figure 2b).

Discussion

In this study, metabolomic analysis was applied to men presenting different semen qualities in order to define relationships between metabolites charactering a low/high sperm concentration. Global serum metabolomic profiles from men presenting different semen qualities were shown to be significantly different and the observed difference was demonstrated as being linked to the biological parameter of spermatozoa concentration. Quantitative measurements of chemical contaminants (Perfluorinated compounds, PFCs) were also investigated in these serum samples. The potential correlation between these PFCs levels and sera metabolomic fingerprints will be investigated soon to identify some biomarkers of exposure. From this proof-of-concept study, the emerging field of metabolomics promises potential for understanding the pathogenesis of many diseases. Moreover, as metabolomic fingerprints are expected not only to reflect composition of endogenous metabolites but pattern of exogenous compounds present in biological fluids through dietary and/or environmental exposure, it promises as well the possibility to understand how chemicals and other environmental stressors can affect both human and wildlife species. However, detection of crucial disturbances in the concentration of key biomarkers can be confounded by inter- and intra-individual differences. Indeed, the qualitative and quantitative compositions of biological samples are highly variable with respect to various genetic, physiological or environmental factors. Therefore, variations in metabolomic data can arise from several sources which can be attributed to biological but also analytical causes. This makes very challengeable the study of endogenous metabolites signatures in body fluids. If the confounding factors are controlled, then metabolomics can offer the possibility through enhanced fundamental mechanistic studies to understand some relation dose-effect and/or the tricky issue of exposure to complex mixtures of chemicals. On a larger scale, the opportunity exists to detect metabolomic disturbances in sentinel species that would signal the potential for ecosystem disturbance before it happens. For human and wildlife populations exposed to contaminants or other environmental stressors, the metabolomic profile offers the potential to detect biomarkers that would indicate not only that exposure has occurred but also that there is a potential biological consequence of that exposure[3]. In conclusion, metabolomics can really be a step-forward in the investigation of environmental contaminants effects on organisms provided analytical and biological issues that would be bias in the results are controlled.

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