RBMO

SHORT COMMUNICATION

Refining the seminal biomarker detection: metabolome profiles before and after the liquefaction procedure

BIOGRAPHY

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ABSTRACT

Research question: Should seminal metabolites be analysed from fresh ejaculate or after liquefaction to establish a protocol for biomarker discovery?

Design: Semen samples were collected from 15 healthy donors, with two aliquots obtained for each donor, one before and one after the liquefaction process, resulting in total of 30 samples for analysis. Non-targeted metabolomics analysis was conducted using liquid chromatography–high-resolution mass spectrometry on these paired samples. Data quality was assessed using MarkerView software. Metabolites were identified using the 2021 NIST Mass Spectral Library, PeakView, CEU Mass Mediator and Sirius software.

Results: A total of 1664 mass-to-charge ratio values were detected and 76 metabolites were identified, including amino acids, lipids, carbohydrates and compounds related to oxidative stress and sperm function. Principal component analysis did not reveal any statistically significant differences between the pre- and post-liquefaction samples. However, univariate statistical testing detected subtle changes in metabolite levels, most (1611) having similar or increased intensities in post-liquefaction samples, along with notable interindividual variability.

Conclusions: The semen liquefaction process does not seem to affect the overall metabolic profile, allowing flexibility in sample analysis without compromising data integrity. This supports the robustness of metabolomics for semen analysis and its potential for identifying new fertility biomarkers.

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Declaration: The authors report no financial or commercial conflicts of interest.

KEY WORDS
Biomarker
Male infertility
Metabolite
Metabolome
Semen quality

INTRODUCTION

Infertility is a frequent chronic disease, affecting around 12–17% of reproductive-aged couples globally, with male-related factors accounting for about 50% of cases (*Fauser et al., 2024*). Despite ongoing efforts to elucidate the pathophysiology of male infertility, some men still receive unexplained infertility diagnoses. Furthermore, traditional semen quality assessment analyses spermatozoa count and motility, but lacks functional insights into fecundity potential (*WHO, 2021*). Therefore, there has been great interest in developing new analytical methods and identifying biomarkers that could predict semen fecundity potential and enhance the understanding of (in) fertility beyond sperm parameters.

With technological advancements, metabolomics emerges as a powerful tool, offering the potential to elucidate metabolic disparities linked to (in)fertility and potentially revealing new therapeutic pathways (*Li et al., 2020*; *Oluwaloseyi et al., 2024*). Nevertheless, to fully harness the potential of metabolomics, a detailed analysis protocol must be established, starting with the optimal timing for semen metabolite analysis: fresh versus post-liquefaction sampling. Liquefaction is a routine process in the clinic, where within 15–20 min post-ejaculation semen transitions into a more liquid state, facilitating its further assessment (*WHO, 2021*). Semen quality is analysed at that phase, but investigation is still needed on whether metabolic markers could be assessed simultaneously with classical quality assessment or instant fresh sample analysis is required. This study set out to compare the metabolome profiles in paired semen samples before and after the liquefaction process using non-targeted metabolomics analysis.

MATERIALS AND METHODS

The study population comprised 15 healthy donors from the Gametia Sperm Biobank (Granada, Spain) selected between January and February 2024 (see Supplementary Table 1 for the semen parameters). The participants maintained a sexual abstinence period of 3–5 days before self-collecting seminal samples at the Biobank. Volumes of 200 μl of each sample (both seminal fluid and sperm cells) were retrieved within first 5 min, while the remaining sample was placed in an incubator at 37°C to achieve liquefaction. After 30 min, a second aliquot was taken. Both aliquots were snap-frozen in liquid nitrogen and stored at −80°C. The study was approved by the Ethics Committee of the University of Granada (CEIM/CEI 0463-M1-18r, approval date 12 July 2018.) The participants provided their written informed consent to participate in the study.

A volume of 100 mg of each sample was processed to isolate the metabolites and remove cell debris and proteins. Each sample was mixed with an internal standard solution containing decanoylcarnitine-d3 (1000 ppb), lysophosphatidylcholine-d7 (500 ppb) and tryptophan-d5 (500 ppb), used for area correction to improve the accuracy of metabolite quantification. This solution was added to each sample in a volume of 2 μl to enhance the quantification accuracy. The samples were then treated with an 80/20 methanol/water solution in a volume ratio of 12:1 methanol-to-sample to separate the metabolites from the larger biomolecules. A blank was processed to remove contaminants from the analysis, and a pooled quality control sample was prepared by combining aliquots from each sample to ensure the method's accuracy and precision. The quality control samples were used to assess signal drift and feature filtering throughout the analytical process.

Sperm cells were disrupted using a FastPrep-24 5G homogenizer (MP Biomedicals, Fisher Scientific, USA) and centrifuged at 17000*g* for 15 min at 10°C. The supernatant containing the metabolites was transferred, evaporated and reconstituted in a solution of 30% methanol, 30% acetonitrile and 40% Milli-Q water (Millipore, Merck KGaA, Germany), achieving a dilution ratio of 2.4. Finally, the samples were filtered using Verex Filter Vials (0.2 μm; Phenomenex, Italy) and stored at 4°C.

Analysis was performed using an Agilent 1290 high-performance liquid chromatography (HPLC) analyser (Agilent Technologies, Germany) coupled with a TOF 5600 mass spectrometer (SCIEX, USA). Two aliquots of each processed sample were analysed: one under positive electrospray ionization mode with a C18 column (Atlantis T3, 2.1 × 150 mm, 3 μm particle size, Waters, Ireland) for non-polar compounds, and another under negative electrospray ionization mode with an HILIC column (XBridge BEH Amide, 2.1 × 150 mm, 2.5 μm particle size; Waters, Ireland) for polar compounds. The mobile phases consisted of water, acetonitrile and 0.1% formic acid, with phase A at 90% water and 10% acetonitrile, and phase B at 10% water and 90% acetonitrile. The flow rates were 300 μl/min and 330 μl/min, with stop times of 20 min and 15 min, respectively. The injection volume for both analyses was 3 μl (for the detailed protocol, see *González–Olmedo et al., 2024*).

The obtained data quality was verified using principal component analysis (PCA) in MarkerView software (version 1.2.1, AB SCIEX, USA). Retention time and mass-to-charge ratio (*m/z*) variability were evaluated with PeakView software (version 1.1.2, AB SCIEX) to correct the peak alignment. Data processing included peak detection and filtering using MarkerView. Signals from impurities and those with a coefficient of variation greater than 30% in the quality control samples were eliminated. MetaboAnalyst (version 5.0, Xia Lab, McGill University, Canada) was employed for the statistical analysis.

The metabolites were annotated following the Metabolomics Society's guidelines, achieving level 2 confidence for identification. Annotation was based on matching the retention time, *m/z*, mass error and fragmentation patterns with the following reference databases: the NIST/EPA/NIH Mass Spectral Library 2021 (version 20, National Institute of Standards and Technology, USA), PeakView software, CEU Mass Mediator (version 3.0, CEMBIO, CEU San Pablo University, Spain) and Sirius software (version 5.8.6, University of Jena, Germany).

Paired t-tests were performed to compare the metabolic profiles of each individual at the pre- and post-liquefaction phase, and a supervised model was applied using partial least squares discrimination analysis (PLS-DA) with permutation testing using 1000 repetitions. In addition, metabolic profiles were generated by calculating the average signal intensities of all the identified metabolites for each group, allowing for a global comparison of the metabolic changes resulting from the liquefaction process. Interindividual variability was assessed using a covariance-adjusted linear model in MetaboAnalyst, applying an analysis of variance style.

RESULTS

A total of 1664 *m/z* values were detected with two analytical approaches and 76

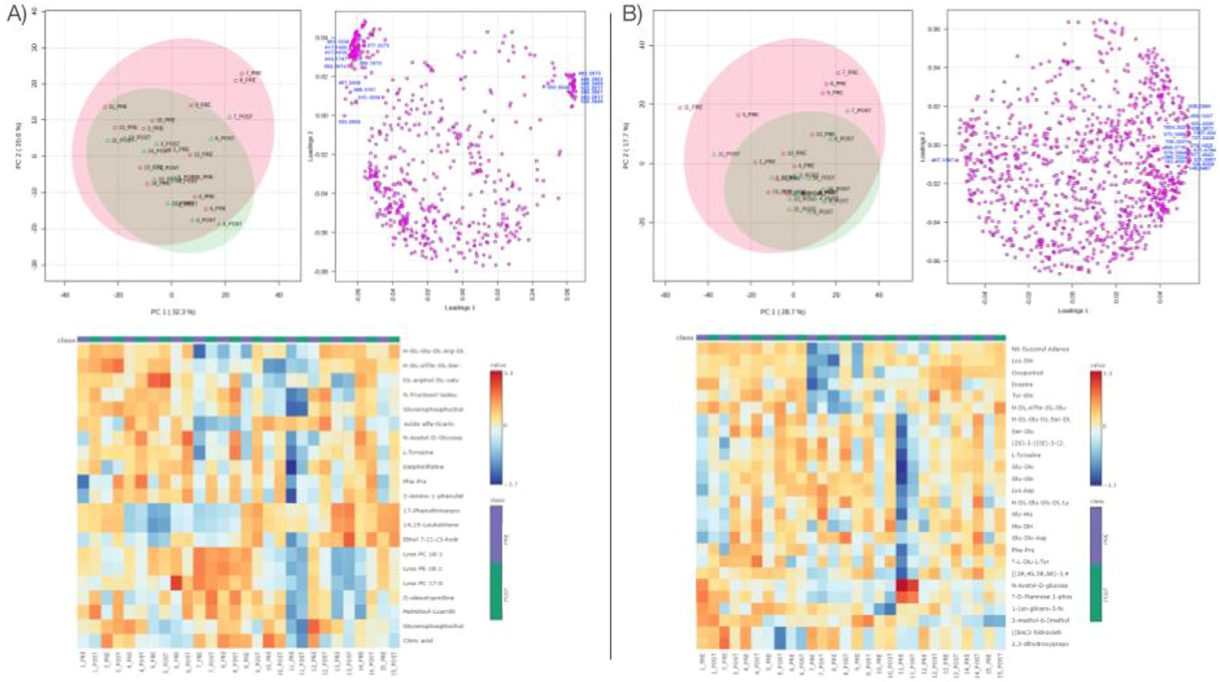

FIGURE 1 Principal component analysis (PCA), loading plots and heatmaps from the analysis in the two ionization modes: (A) positive ionization mode and (B) negative ionization mode. The PCA plots show metabolomic differences between the pre-liquefaction (PRE, red/purple) and post-liquefaction (POST, green) samples, with confidence ellipses for each group. The top 20 *m/z* values contributing to the model are shown in the loading plots. Identified metabolites include lysophosphatidylcholine (LPC) (19:1) at *m/z* 550.3849 and lysophosphatidylethanolamine (LPE) (18:1) at *m/z* 480.3052. The heatmaps display the abundance of the metabolites. This analysis was performed on paired semen samples using liquid chromatography–high resolution mass spectrometry.

metabolites were identified (Supplementary Table 2). Statistical analysis revealed subtle variations in metabolite levels between all the samples (FIGURE 1). Notably, 31.6% (525) of the detected *m/z* values, corresponding to metabolites, showed significant differences between the pre- and post-liquefied samples. However, only 3.18% (53) of the evaluated molecules exhibited higher intensities before liquefaction, with fold change values of over 1.5. The remaining metabolites were detected with similar or increased intensities in the post-liquefaction samples (1611), probably due to the effect of increasing osmolality over time (*Holmes et al., 2019*).

On the other hand, upon PCA analysis, the pre- and post-liquefaction samples did not show any clear differences (FIGURE 1), indicating that there were no significant differences in the metabolic profiles between the samples. In addition, the permutation test was not significant for the PLS-DA model (Supplementary Figure 1). An analysis of interindividual variability revealed considerable differences across the samples, with 1474 significant *m/z* values (FDR < 0.05), of which 201 remained significant after multiple correction.

DISCUSSION

Metabolomic analysis holds great promise for identifying novel biomarkers of male (in)fertility potential as it can reveal subtle metabolic alterations that are not detectable by conventional methods (*Li et al., 2020*). The incorporation of such analyses into routine semen evaluation could improve the diagnosis of infertility and lead to targeted and effective treatments (*Oluwaloseyi et al., 2024*).

This pilot study investigated the impact of the semen liquefaction process on the semen's metabolic profile for developing a protocol for biomarker identification. The study results demonstrate considerable interindividual variability in metabolite concentrations, highlighting the complexity of semen biochemistry and need for protocol unification (*Li et al., 2020*). When comparing the pre- and post-liquefaction samples, no statistically significant differences in overall metabolic profiles were detected, indicating that the timing of the sample analysis does not seem to alter the power for detecting the metabolites. The absence of compromised metabolic integrity in the post-liquefaction samples simplifies the potential metabolite biomarker analysis, enabling the possibility to integrate it into the routine semen quality check-up rather than requiring instant ejaculate processing. Altogether, the study results support the robustness of metabolomics for semen analysis and underscore its potential for developing new biomarkers of (in)fertility and fecundity.

ACKNOWLEDGMENTS

The authors thank all the cohort members and researchers who participated in the study. This work is part of a PhD thesis conducted in Biomedicine Doctoral Studies at the University of Granada, Spain.

FUNDING

This work is supported by Grants Endo-Map (PID2021-12728OB-100) and ROSY (CNS2022-135999) funded by MICIU/AEI/https://doi.org/10.13039/501100011033 and by FEDER, EU; and Grant FPU23/01576 funded by MCIN/AEI/https://doi.org/10.13039/501100011033 and by ESF Investing in Your Future. This work is also

supported by the Metabolomics Workbench/National Metabolomics Data Repository (NMDR) (grant no. U2C-DK119886), Common Fund Data Ecosystem (CFDE) (grant no. 3OT2OD030544) and Metabolomics Consortium Coordinating Center (M3C) (grant no. 1U2C-DK119889).

DATA AVAILABILITY

The data are available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench (https://www.metabolomicsworkbench.org), where it has been assigned Project ID MTBLS11207. The data can be accessed via the following link: https://www.ebi.ac.uk/metabolights/MTBLS11207.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2025.104856.

REFERENCES

Fauser, B.C.J.M., Adamson, G.D., Boivin, J., Chambers, G.M., Dyer, S., de Geyter, C., Inhorn, M.C., Schmidt, L., Serour, G.I., Tarlatzis, B., Zegers-Hochschild, F., 2024. Declining global fertility rates and the implications for family planning and family building: an IFFS consensus document based on a narrative review of the literature. Hum Reprod Update 30, 153–173. https://doi.org/10.1093/humupd/dmad028.

González-Olmedo, C., García-Verdejo, F.J., Reguera-Teba, A., Rosa-Garrido, C., López-López, J.A., Díaz-Beltrán, L., García, P.M., Luque-Caro, N., Gálvez-Montosa, F., Ortega-Granados, A.L., Ruiz-Sanjuan, M., Cózar-Ibáñez, A., Sainz, J., Marchal, J.A., Camacho, J., Del Palacio, J.P., Fernández-Godino, R., Díaz, C., Sánchez-Rovira, P., 2024. Metabolomics signature as a survival predictor in patients with resectable colorectal liver metastasis. Clinical and Translational Medicine 14 (1), e1541. https://doi.org/10.1002/ctm2.1541.

Holmes, E., Björndahl, L., Kvist, U., 2019. Post-ejaculatory increase in human semen osmolality in vitro. Andrologia 51 (7), e13311. https://doi.org/10.1111/and.13311.

Li, L., Hao, X., Chen, H., Wang, L., Chen, A., Song, X., Hu, Z., Su, Y., Lin, H., Fan, P., 2020. Metabolomic characterization of semen from asthenozoospermic patients using ultra-high-performance liquid chromatography–tandem quadrupole time-of-flight mass spectrometry. Biomedical Chromatography 34, 1–9. https://doi.org/10.1002/bmc.4897.

Oluwaloseyi, A., Aduragbemi Noah, O., Lydia Oluwatoyin, A., Gaffar, Y., Moses, O., Oyedayo Phillips, A., Comfort Onaolapo, M., Sylvester Olateju, B., Ademola Ayodele, A., Mega Obukohwo, O., Ayodeji Folorunsho, A., 2024. Metabolomics of male infertility. Clinica Chimica Acta 556, 117850. https://doi.org/10.1016/j.cca.2024.117850.

World Health Organization, 2021. WHO laboratory manual for the examination and processing of human semen, Sixth edition World Health Organization Press. https://www.who.int/publications/i/item/9789240030787.

Received 2 October 2024; received in revised form 28 January 2025; accepted 29 January 2025.