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Full length article

Seminal plasma metabolomics profiles following long (4–7 days) and short (2 h) sexual abstinence periods

H. Alipour [a,*], R.K. Duus [a], R. Wimmer [b], F. Dardmeh [a], S.S. Du Plessis [c,d], N. Jørgensen [e], O.B. Christiansen [f,g], C. Hnida [f], H.I. Nielsen [a], G. Van Der Horst [h]

[a] Department of Health Science and Technology, Regenerative Medicine Group, Aalborg University, Aalborg, Denmark
[b] Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark
[c] Department of Basic Medical Sciences, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates
[d] Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
[e] University Department of Growth and Reproduction and International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, Copenhagen, Denmark
[f] Department of Obstetrics and Gynecology, Fertility Unit, Aalborg University Hospital, Aalborg, Denmark
[g] Institute of Clinical Medicine, Aalborg University, Denmark
[h] Department of Medical Biosciences, University of the Western Cape, Cape Town, South Africa

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ABSTRACT

Objective: Metabolomic profiling of seminal plasma has been suggested as a possible approach for a fast and non-invasive male infertility evaluation diagnosis. However, metabolomics profiles in normozoospermic men have not been thoroughly investigated, and the influence of ejaculation-abstinence has not been described.

To provide interim reference values and find associations between the metabolomics profiles of human seminal plasma and length of ejaculation-abstinence period in normozoospermic men.

Study design: Semen samples collected after long (4–7 days) and short abstinence (2 h) from 31 normozoospermic males were assessed for routine quality parameters before the seminal plasma was separated by centrifugation. Metabolomics profiles of the seminal plasma were then determined using untargeted Nuclear Magnetic Resonance Spectroscopy.

Results: In total, 30 metabolites were identified. Pyruvate showed a higher concentration, while fructose, acetate, choline, methanol, N-acetylglucosamine, O-acetylcarnitine, uridine, and *sn*-glycero-3-phosphocoline showed lower concentrations in samples collected after short abstinence (vs. long). All metabolites showed lower absolute amounts (volume × concentration) following shorter abstinence. However, the lower sperm concentration in samples collected after short abstinence resulted in higher absolute amounts of pyruvate and taurine per spermatozoa: pyruvate 1.92 (1.12–3.87) vs. 1.29 (0.83–2.62) ($P < 0.001$) and taurine 0.58 (0.36–0.92) vs. 0.43 (0.28–0.95) ($P < 0.05$) ng/10^6 spermatozoa. Simultaneously, there was a higher percentage of progressively motile spermatozoa in samples collected after the short abstinence.

Conclusion: The generally lower concentrations of seminal metabolites after short abstinence periods may be related to the shorter time available for secretion and collection of these metabolites by the accessory glands and the epididymides. The concomitant lower number of spermatozoa in the second ejaculate resulted in increased absolute amounts of pyruvate and taurine per spermatozoa, accompanied by increased spermatozoa motility in these samples.

The simultaneous increase in percentages of motile spermatozoa and absolute amounts of pyruvate and taurine per spermatozoa after shorter abstinence might indicate that these two metabolites play a more critical role in sperm motility, which should be further investigated in future studies.

Abbreviations: HSP, human seminal plasma; NMR, Nuclear Magnetic Resonance; IVF, in-vitro fertilization; ICSI, intra-cytoplasmic sperm injection; SCA, Sperm Class Analyzer; FID, free induction decay.

* Corresponding author.
E-mail address: hiva@hst.aau.dk (H. Alipour).

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Introduction

Male-factor infertility contributes to almost half of the cases with couples' infertility [1]. Investigations of infertile men focus on assessing classical parameters like sperm concentration, motility, and morphology. Assessment of seminal plasma is only done in severe cases where the concentration of accessory glands secretions (e.g. fructose, citric acid, α-glucosidase or zinc) are measured to assess their function [2].

In humans, seminal plasma (HSP) does not progress beyond the external cervix [3], and it is considered the nutritious and protein-rich environment needed for spermatozoa to function [4]. However, HSP may affect the fertilization ability and even successful implantation and pregnancy outcomes [3]. The multifocal origin of seminal plasma (~65% from the seminal vesicles, ~25% from the prostate and ~10% from testes and epididymides, and ~1% from the bulbourethral and periurethral glands) has complicated the search for biomarkers in this fluid [5].

Identifying the metabolome may improve our understanding of cells' normal composition, physiology, and dysfunction [6-9]. Spectrometric and chromatographic methods can be used to assess metabolomic profiles [10,11]. The non-destructive, non-selective, non-biased, quantitative ability to identify novel compounds via their unique spectral patterns has made Nuclear Magnetic Resonance (NMR) spectroscopy a particularly appealing tool for metabolomics [12].

In contrast to less than 100 metabolites, over 6000 proteins in the human spermatozoa (about 80% of this cell's estimated proteome) have been identified[13]. In general, humans have a much lower number of metabolites than proteins [8,14]. Despite the notable developments in metabolomics technology, the relatively lower number of metabolites identified in sperm and HSP may be because the 'omics' techniques have not been applied to their full potential in this field [4,6,13].

Nonetheless, metabolomic profiling of seminal plasma has been suggested as a possible approach for a fast and non-invasive diagnosis in the evaluation of male infertility [7,15-17]. Studies assessing HSP metabolomics profiling by LC-MS/MS as a screening tool for male infertility demonstrated a correlation between HSP metabolites and sperm concentration and morphology [18]. NMR profiling of HSP metabolites combined with Principal Component Analysis (PCA) has been able to differentiate semen quality and classify patients with leukocytospermia, varicocele, testicular cancer, necrozoospermia, azoospermia [19], and oligoasthenoteratozospermia [20]. A more recent comparative study using ^{1}H NMR could not classify teratozoospermia index (TZI) using HSP metabolite profiling in fertile and teratozoospermia patients [21].

A gap in our knowledge regarding HSP metabolites and their impact on semen quality and function still exists. Namely, changes in HSP metabolomics profile following different abstinence periods in the same person have not been yet determined. Therefore, this exploratory study focused on characterizing and comparing the HSP metabolomics profile in two consecutive ejaculates collected from normozoospermic males after long (4–7 days) followed by a short (≤2 h) ejaculation-abstinence period to provide interim reference values and find associations between the metabolomics profiles of human seminal plasma and length of ejaculation abstinence period in normozoospermic men.

Materials and methods

Ethical approval

The samples were collected at Dronninglund Fertility Clinic (Aalborg University Hospital) following approval by the scientific ethics committee of the North Jutland Region, Denmark (approval number N-20140023). Informed written consent was obtained from all subjects, and the study was performed in accordance with the Declaration of Helsinki as revised in Tokyo 2004.

Study subjects

The study group consisted of thirty-one male partners from couples undergoing in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) regardless of the etiology. Their median (Interquartile range (IQR)) age was 33.5 (30–39) years, and median body mass index (BMI) was 26.5 (23.5–28.3). To not interfere with the treatment process on the day of the study, only men with a minimum sperm concentration of 15 million/ml (WHO reference value) in the first ejaculate were included in the study. Participants were without a medical history of vasectomy, orchitis, unilateral orchiectomy, ejaculatory disorders, diabetes, cardiovascular disease, medically treated psychological illness, endocrine, or genetic diseases.

Sample collection

All participants had an ejaculation-abstinence period of 4–7 days (median (IQR) of 6 (5–7) days) before delivering the semen sample for IVF/ICSI treatment. The second semen sample was delivered 2 h after the first. All samples were obtained by masturbation into a wide-mouthed plastic container and allowed to liquefy (~60 min) at room temperature. Semen volume was measured using a graduated pipette, and 1000 μL from each sample was used for seminal plasma collection.

Semen analysis, Sperm Class Analyzer (SCA®)

The Motility/Concentration module of the SCA® (version 5.4; Microptic S.L., Barcelona, Spain) connected to a Basler Scout A780–54fc camera (Basler, Germany) capturing at 50fps, mounted on a Nikon Eclipse 50i microscope with a 10x positive phase contrast lens (Nikon, Japan) was used to determine sperm concentration and progressive motility as previously described [22]. Five different fields along the middle of a Leja 10 (Leja, Netherlands) slide loaded with 2 μL of well-mixed neat semen were evaluated for each sample to detect and compensate for any possible non-uniform distribution of spermatozoa in the chamber [23]. Spermatozoa were classified as progressively motile, non-progressive motile or immotile [24]. Sperm morphology was assessed using the morphology module of the SCA®as previously described [25].

Nuclear magnetic resonance spectroscopy

After liquefaction, 1 ml of the semen sample was centrifuged at 3000×*g* for 20 min at 4 °C to separate the seminal plasma from the sperm. The top 500 μL of the supernatant was collected and immediately frozen in sterile Eppendorf tubes (Eppendorf, USA) at −80 °C. Frozen samples were thawed slowly on ice (~ 10 min) and centrifuged at 12,100×*g* for 30 min to remove the remaining cell debris. The supernatant collected from each sample was vortexed for 10 s. 100 μL of supernatant was added to 420 μL D_2O and 60 μL 0.5 M phosphate buffer pH 7 containing 0.23 M TSP-d_4 (sodium-3-trimethylsilyl-[2,2,3,3-$^{2}H_4$]-propionate) and 20 mM NaN_3. Subsequently, pH was adjusted to 7.0 ± 0.1 using a BRUKER BT pH titrator. 550 μL of the sample were transferred to a 5 mm NMR tube. The samples were continuously kept on ice between steps.

^{1}H NMR spectra were acquired on a Bruker AVIII-600 MHz NMR spectrometer (Bruker Biospin, Switzerland) with a CPP-TCI probe. Topspin 3.2pl5 (Bruker Biospin, Germany) was used for acquiring

spectra at 310.1 K. CPMG (T_2 relaxation-edited Carr–Purcell–Meiboom–Gill [26] (128 scans, 114940 complex data points, spectral width of 23.94 ppm, acquisition time 4 s, relaxation delay 0.97 s. Weak continuous wave irradiation ($\gamma B_1/2\pi$ = 46.6 Hz) was used for water suppression. The total spin-echo relaxation delay was 80.17 ms. This consisted of 130 repetitions of (τ–π–τ) sandwiches (τ = 0.3 ms and π is a 180° pulse of ≈16.7 μs. For the conformation of metabolites, ^{1}H–^{1}H 2D-TOCSY (Total Correlation Spectroscopy) with a 120 ms clean-TOCSY mixing sequence and a multiplicity-edited ^{1}H–^{13}C HSQC (Heteronuclear Single Quantum Coherence) spectra were recorded on selected samples.

Chenomx NMR suite 8.1 (Chenomx, Canada) was used for the identification and quantification of metabolites. The methyl group signals of TPS-d_4 were used as internal concentration standard. Apart from the built-in ChenomX library, the Human Metabolome Database [8,27,28] was used to search for matching compounds and verify resonance assignments.

Statistical analysis

Metabolite concentrations (mg/dl) are shown as medians (IQR). The Shapiro–Wilk normality test was applied, and data with non-normal distributions were corrected by natural logarithmic or cubic-root transformation. A paired-samples *t*-test was used to compare the metabolite concentrations and sperm motility results of consecutive ejaculates from individual men. Semen volumes are reported as medians (IQR) and compared using the "Wilcoxon matched pairs" test as the transformations did not result in a normal distribution. The association between abstinence duration, semen volume, sperm concentration, and metabolite concentrations in the first and second ejaculates, and the changes of the mentioned parameters between the first and second ejaculates (Ej.2–Ej.1) were assessed using Spearman's non-parametric correlation. Statistical analyses were performed using MedCalc® software version 15.8 (MedCalc Software, Belgium).

Results

Table 1 shows semen parameters for samples collected after 4–7 days and 2 h of abstinence, and the main finding of this has been published previously [22]. A typical within-subject comparison of NMR spectra of seminal plasma from ejaculates collected after long (4–7 days; blue spectra) and short (2 h; red spectra) ejaculatory abstinence can be seen in Fig. 1. A total of 30 metabolites (16 Amino acids, four energy metabolites, and ten other metabolites) were identified and quantified. Table 2 shows the concentrations, absolute amounts, and absolute amounts per 10^6 spermatozoa (absolute amounts/total sperm count) of the metabolites.

Among the 30 identified metabolites, pyruvate had a higher concentration in samples obtained after short ejaculation-abstinence, while fructose, acetate, choline, methanol, N-acetylglucosamine, O-acetylcarnitine, uridine, and *sn*-glycero-3-phosphocoline had lower concentrations. There was no difference for the remaining 21 metabolites (Table 2). All metabolites showed lower absolute amounts in the second ejaculate. Pyruvate and tau-

Table 1
Median and interquartile ranges (IQR) of semen volume, sperm concentration, total motile sperm concentration, total sperm count (TSC), total motile sperm count (TMSC) and percentage of sperm categorized based on progression (WHO 2010), using the SCA in samples (Immotile: Non-motile sperm, NPM: Non-progressive motile sperm; PM: Progressive motile sperm) collected after 4–7 days (ejaculate 1) and 2 h (ejaculate 2) of abstinence (N = 31). P values demonstrate significant differences between the two ejaculates.

	Semen volume (ml)	Total Count ($\times 10^6$) Median (IQR)		Concentration (Mill/ml) Median (IQR)		Progressive motility Median (IQR)		
		Total sperm count	Total motile sperm count	Sperm concentration	Total motile sperm concentration	*PM %*	*NPM %*	*Immotile %*
Ejaculate 1	3.0 (2.4–3.8)	114 (76–169)	81 (45–122)	40 (28–56)	23 (15–40)	38 (20–54)	24 (21–30)	31 (22–52)
Ejaculate 2	1.8 (1.5–2.5)	60 (38–89)	43 (28–62)	33 (19–46)	23 (15–38)	47 (33–61)	25 (19–33)	26 (14–34)
P value	<0.0001*	<0.0001*	<0.0001*	<0.05*	0.31	<0.001*	0.831	<0.001*

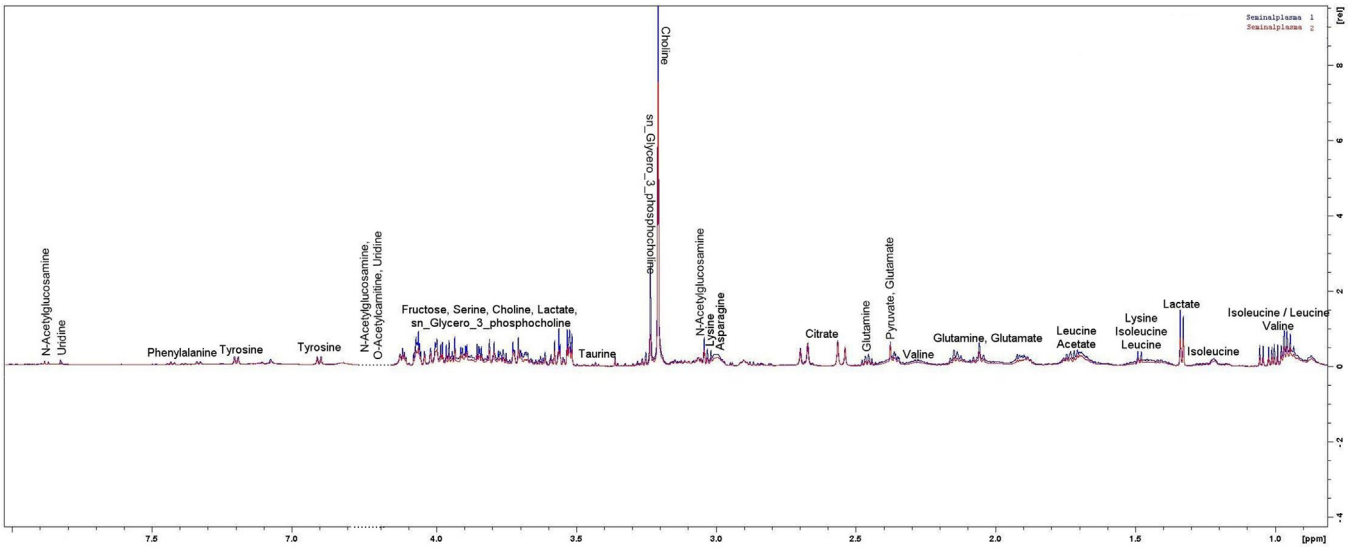

Fig. 1. Typical within-subject comparison of NMR Spectra of Seminal plasma from ejaculates collected after long (4–7 days; blue spectra) and short (2 h; red spectra) ejaculatory abstinence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Median and interquartile ranges (IQR) of concentrations [mg/dl] of seminal plasma metabolites (corresponding to a dilution factor of 6) identified by ^{1}H-nuclear magnetic resonance spectroscopy, absolute amounts (volume X concentration) of metabolites, and absolute amounts (ng) of metabolites per 1000 spermatozoa in samples collected after 4–7 days (ejaculate 1) and 2 h (ejaculate 2) of sexual abstinence (N = 31). * demonstrates pairwise significant differences.

Metabolites				Concentrations [mg/dl]Median (IQR)			Absolute amounts (volume × metabolite concentration) [mg]Median (IQR)			Absolute amounts per 10^6 spermatozoa (absolute amount/total sperm count) [ng/10^6 spermatozoa]Median (IQR)		
				Ejaculate 1	Ejaculate 2	P value	Ejaculate 1	Ejaculate 2	P value	Ejaculate 1	Ejaculate 2	Pairwise significance
Amino acids	Non-essential	**1**	Alanine	6.75 (4.88–8.44)	4.89 (3.63–6.07)	0.72	0.21 (0.12–0.24)	0.09 (0.07–0.14)	<0.0001*	1,50 (1.09–2.79)	1.52 (0.90–3.33)	0.62
		2	Arginine	8.55 (5.95–12.18)	6.39 (3.93–7.637)	0.75	0.26 (0.17–0.37)	0.10 (0.09–0.19)	<0.0001*	2.18 (1.48–3.33)	1.96 (1.04–3.20)	0.26
		3	Asparagine	9.94 (7.99–13.23)	7.15 (5.44–9.43)	0.72	0.31 (0.20–0.39)	0.13 (0.10–0.21)	<0.0001*	2.13 (1.71–4.28)	2.39 (1.34–4.27)	0.54
		4	Glutamate	22.15 (17.47–27.97)	16.33 (12.52–20.23)	0.74	0.68 (0.46–0.87)	0.31 (0.22–0.48)	<0.0001*	5.39 (3.55–8.61)	4.92 (3.68–12.80)	0.69
		5	Glutamine	18.14 (14.16–22.47)	13.94 (8.32–17.43)	0.77	0.51 (0.34–0.67)	0.22 (0.17–0.38)	<0.0001*	4.35 (2.281–7.71)	4.06 (2.32–5.61)	0.62
		6	Glycine	12.68 (8.54–18.10)	8.78 (5.83–12.30)	0.69	0.40 (0.26–0.51)	0.17 (0.13–0.23)	<0.0001*	2.77 (2.17–5.80)	2.79 (1.60–6.92)	0.41
		7	Proline	2.71 (1.73–3.75)	2.48 (1.10–3.25)	0.1	0.29 (0.19–0.39)	0.12 (0.09–0.20)	0.0002*	2.22 (1.69–4.17)	2.21 (1.28–4.06)	0.91
		8	Serine	38.32 (28.51–52.91)	28.49 (21.88–37.12)	0.65	0.40 (0.26–0.56)	0.17 (0.12–0.29)	<0.0001*	3.01 (2.19–5.31)	3.22 (1.77–5.51)	0.59
		9	Tyrosine	13.03 (11.12–16.66)	9.78 (7.88–12.07)	0.61	0.28 (0.20–0.54)	0.15 (0.07–0.26)	<0.0001*	2.33 (1.27–4.94)	2.49 (0.92–5.61)	0.42
	Essential	**10**	Isoleucine	10.21 (7.89–12.45)	7.11 (4.73–8.88)	0.76	0.10 (0.07–0.14)	0.05 (0.03–0.08)	<0.0001*	0.74 (0.57–1.25)	0.80 (0.51–1.51)	0.35
		11	Leucine	14.29 (9.83–16.26)	9.25 (6.85–12.08)	0.92	0.08 (0.05–0.12)	0.05 (0.03–0.07)	<0.0001*	0.61 (0.45–1.11)	0.61 (0.30–1.60)	0.90
		12	Phenylalanine	3.51 (2.87–4.32)	2.68 (2.04–3.22)	0.74	1.19 (0.76–1.62)	0.52 (0.37–0.85)	<0.0001*	8.63 (6.58–16.60)	9.04 (5.95–16.2)	0.79
		13	Threonine	7.63 (6.59–10.05)	5.03 (3.01–6.90)	0.66	0.22 (0.16–0.30)	0.08 (0.06–0.16)	<0.0001*	1.76 (1.22–3.18)	1.50 (0.86–3.85)	0.19
		14	Tryptophan	0.22 (0.17–0.36)	0.18 (0.15–0.42)	0.83	0.01 (0.01–0.02)	0.01 (0.00–0.01)	<0.0001*	0.06 (0.04–0.13)	0.08 (0.03–0.31)	0.19
		15	Lysine	9.99 (5.87–18.05)	6.05 (4.26–11.69)	0.75	0.37 (0.27–0.53)	0.19 (0.12–0.28)	<0.0001*	3.20 (2.21–5.04)	3.24 (1.96–10.40)	0.14
		16	Valine	10.53 (7.93–13.77)	7.26 (4.73–9.87)	0.69	0.32 (0.20–0.41)	0.13 (0.09–0.19)	<0.0001*	2.34 (1.71–4.33)	2.03 (1.18–5.05)	0.31
Energy	metabolites	**17**	Fructose	44.95 (32.66–51.51)	34.15 (27.68–46.44)	<0.05*	1.44 (0.80–1.76)	0.73 (0.42–1.15)	<0.0001*	12.2 (6.84–16.90)	12.90 (6.87–17.70)	0.63
		18	Galactose	0.96 (0.70–1.23)	0.92 (0.55–1.20)	0.16	0.03 (0.02–0.04)	0.02 (0.01–0.03)	<0.0001*	0.24 (0.13–0.47)	0.26 (0.17–0.73)	0.16
		19	Lactate	12.24 (9.57–17.49)	10.65 (9.35–15.23)	0.42	0.33 (0.24–0.56)	0.24 (0.17–0.30)	<0.0001*	3.34 (2.65–4.49)	3.86 (2.40–5.69)	0.06
		20	Pyruvate	5.24 (3.92–6.71)	7.05 (5.11–9.14)	<0.001*	0.14 (0.10–0.21)	0.13 (0.06–0.21)	<0.0288*	1.29 (0.83–2.62)	1.92 (1.12–3.87)	<0.001*
	Other metabolites	**21**	Acetate	1.73 (1.49–2.29)	1.65 (1.15–2.19)	<0.01*	0.05 (0.04–0.09)	0.03 (0.02–0.05)	<0.0001*	0.48 (0.34–0.81)	0.53 (0.35–0.95)	0.66
		22	Choline	53.27 (40.82–64.32)	45.1 (32.80–59.79)	<0.01*	1.76 (1.06–2.43)	0.89 (0.57–1.42)	<0.0001*	14.8 (8.91–25.00)	14.4 (9.02–23.60)	0.68
		23	Citrate	29.29 (21.33–36.40)	24.29 (18.74–33.74)	0.08	0.82 (0.66–1.09)	0.49 (0.35–0.71)	<0.0001*	7.13 (4.52–12.00)	9.23 (4.51–19.6)	0.08
		24	Formate	0.08 (0.068–0.10)	0.08 (0.07–0.10)	0.49	[0.24 (0.16–0.33)] $*10^{-2}$	[0.16 (0.10–0.21)] $*10^{-2}$	<0.0001*	0.02 (0.01–0.03)	0.03 (0.02–0.04)	0.05
		25	Methanol	0.81 (0.67–1.26)	0.76 (0.51–1.03)	<0.001*	0.03 (0.02–0.04)	0.01 (0.01–0.02)	<0.0001*	0.25 (0.16–0.42)	0.22 (0.14–0.50)	0.73
		26	N-Acetylglucosamine	5.64 (4.66–7.35)	4.95 (3.25–6.65)	<0.01*	0.18 (0.13–0.25)	0.09 (0.06–0.15)	<0.0001*	1.34 (0.91–2.42)	1.43 (0.85–2.85)	0.86
		27	O-Acetylcarnitine	0.54 (0.42–1.01)	0.49 (0.39–0.79)	<0.05*	0.02 (0.01–0.03)	0.01 (0.01–0.02)	<0.0001*	0.15 (0.10–0.31)	0.18 (0.10–0.33)	0.23
		28	Taurine	1.59 (1.41–2.19)	1.58 (1.27–2.37)	0.39	0.05 (0.04–0.08)	0.03 (0.02–0.05)	<0.0001*	0.43 (0.28–0.95)	0.58 (0.36–0.92)	<0.05*
		29	Uridine	3.91 (3.40–4.77)	3.69 (2.44–4.29)	<0.01*	0.11 (0.08–0.15)	0.07 (0.04–0.10)	<0.0001*	0.95 (0.65–1.77)	0.97 (0.59–1.97)	0.75
		30	*sn*-Glycero-3-phosphocholine	7.25 (6.07–9.47)	5.14 (3.52–8.51)	<0.01*	0.22 (0.16–0.28)	0.11 (0.07–0.17)	<0.0001*	1.97 (1.36–2.99)	1.84 (1.12–3.00)	0.44

rine had higher absolute amounts per 10^6 spermatozoa, whereas this measure was lower for all other metabolites (Table 2). Multivariate statistics (PCA and PLDSA) did not provide satisfying results and are therefore not included.

Discussion

To the best of our knowledge, this is the first study describing the intra-individual differences in metabolomics profiles in HSP of normozoospermic men after short and long abstinence periods.

The identified metabolite variations coincided with higher percentages of motile spermatozoa in ejaculates obtained after the short abstinence [22,29,30]. To which degree these associations reflect causality remains to be shown. Nevertheless, the longer duration of spermatogenesis [31] than the abstinence periods assessed in this study and the lack of any evident variation in morphology between the two samples suggests that the observed difference in quality between the two ejaculates was not associated with spermatogenesis and maturation processes, but possibly with the composition of HSP. It also makes it safe to assume that the percentage of immature sperm was not increased with shorter abstinence.

The metabolomic composition of the HSP is influenced by secretions from the prostate, seminal vesicles, epididymides, testes, bulbourethral and periurethral glands [4,5]. The amino acids in human HSP mainly originate from the testes or epididymides [32] and partly from the proteolytic activity in the semen after ejaculation [33]. Whether metabolite concentrations in the HSP are biologically controlled by a capacity not related to the abstinence period remains unknown. Nevertheless, the decreased concentration of all identified metabolites after the short abstinence period (although insignificant) could be related to insufficient time (2 h) for the collection and secretion of the metabolites by the accessory glands or a shorter period of the proteolytic activity before ejaculation, rather than a change in metabolic pathways. Additional investigations are required to confirm this hypothesis.

A recent meta-analysis revealed that shorter abstinence resulted in higher fertilization rates (P = 0.09) and significantly higher implantation (P = 0.0001) and pregnancy rates (p = 0.006) following fresh embryo transfer cycles for patients undertaking ART [34]. It can be speculated that modest changes in the HSP composition may have affected the spermatozoa motility and kinematic parameters.

Animal studies showed pyruvate and lactate as the most important energy sources for sperm mitochondrial function and motility [35]. Human spermatozoa presented increased intracellular ATP levels, progressive motility and hyperactivation (56, 21 and 130%, respectively) when supplemented with exogenic pyruvate [36]. Despite the lower concentrations of pyruvate and lactate, in the HSP of the second ejaculates, the lower total number of spermatozoa resulted in higher absolute amounts of these energy metabolites per spermatozoa, which may potentially explain the better motility characteristics observed in the second ejaculates. The protective effect of pyruvate against oxidative stress previously shown in somatic cells [37,38] and sperm of cyclophosphamide-treated mice [39] may have also played a role towards the better quality of the sperm in the second ejaculates.

Taurine is an essential amino acid that is not grouped with the other amino acids since it is not a protein building block [8]. The exact origin of taurine in human semen is unclear, but animal studies suggest an epididymal origin [40]. Taurine is important as a capacitating agent [41,42] and a sperm motility factor [43,44] with antioxidative and membrane-stabilizing effects [21,44-47]. Low taurine levels in seminal plasma and sperm have been suggested as markers of male factor infertility [47]. Thus, the observed higher level of taurine per spermatozoa might have also had a role in the better Motility of the sperm in the second ejaculates.

Limitations and strengths

Untargeted NMR metabolomics offers the advantage of an unbiased global analysis of all small molecules in a given body fluid. The study is thus not limited to a set of predefined metabolites tested for, but every metabolite present above a certain concentration threshold can be identified and quantified. At the same time, sample preparation and pretreatment are minimal for NMR investigations. The weakness of the NMR method is the rather high metabolite concentrations required. Metabolites present at concentrations <20 μM are likely not detectable with our experimental setup, and even metabolites present at higher concentrations can easily be missed if their resonances are obscured by resonances of metabolites present in higher concentrations.

Missing some metabolites, such as Histidine, methionine, aspartic acid (aspartate), and cysteine that had been identified in the HSP by previous studies [49,50], could be due to technical issues regarding the procedure of identifying the many extensively overlapping resonances in a single pulse spectra produced by untargeted whole seminal fluid ^{1}H NMR [48]. It is also possible that essential amino acids originating from the diet are not present in the accessory sex glands, while non-essential amino acids are absorbed in the epididymis or not even synthesized by the accessory sex glands. Nevertheless, the reason why some metabolites were not identified remains debatable.

It has to be kept in mind that the possible elemental exchange between HSP and sperm cells, possibly impacted by sperm concentration, may also be a co-factor complicating the interpretation of the metabolite measurements [49]. Further studies eliminating the possible effect of sperm metabolism after ejaculation and during liquefaction time can exclude some confounding factors and deliver a better explanation for the underlying mechanism of changes in the metabolites concentrations and its effect on sperm quality in consecutive ejaculates. Ensuring the lack of contamination of HSP with cell debris by filtration of the HSP after centrifugation [50] could also assure more precise results in future studies.

Conclusion

Nuclear Magnetic Resonance Spectroscopy detected lower concentrations of seminal plasma metabolites except for pyruvate in ejaculates collected after short abstinence periods, which may be related to the shorter time available for the secretion and collection of these metabolites by the accessory glands. However, the concomitant lower sperm concentration in the second ejaculate resulted in increased absolute amounts of pyruvate and taurine per spermatozoa, accompanied by better sperm motility in these samples. The reported metabolite concentrations may be used as interim reference values for short and long abstinence periods and comparison by future studies. Further investigation on the complex composition of the metabolites in the seminal plasma and their possible effect on sperm function is required before a definite conclusion regarding a potential causality between HSP metabolite concentrations, and sperm quality can be reached.

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S/L (Barcelona, Spain) on several occasions. All authors declare no conflict of interest.

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