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Metabolomic analysis of seminal fluids in infertile individuals

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Abstract. – OBJECTIVE: Infertility impacts a substantial number of couples worldwide, and about 50% of cases are linked to male factors. The analysis of seminal fluid composition can improve diagnostic accuracy and offer deeper insights into the pathophysiology of male factor infertility. This study seeks to identify novel markers for diagnosing and treating male infertility by comparing organic acid profiles in the seminal fluid of individuals with normospermia, oligospermia, and azoospermia.

PATIENTS AND METHODS: Semen samples were collected from men with normospermia, oligospermia, and azoospermia. The organic acid profile in the seminal fluid was analyzed using liquid chromatography-mass spectrometry/mass spectrometry (LC/MS-MS). Data analysis was performed using SPSS and MetaboAnalyst.

RESULTS: The study revealed significant differences in metabolite levels among normospermic, oligospermic, and azoospermic individuals. In groups with oligospermia, there were significant decreases in the levels of 2-OH-Isovaleric Acid, 3-Methyl-2-Oxovaleric Acid, Ethyl-Malonic Acid, Citric Acid, Oxoproline, Malic Acid, N-Acetyl-Aspartic Acid, Suberic Acid, Glutaconic Acid, and Succinic Acid. Similarly, individuals with azoospermia exhibited a notable reduction in the levels of Citric Acid, Malic Acid, and Suberic Acid. Furthermore, according to the Variable Importance in the Projection (VIP) score analysis, Ethyl-Malonic Acid, Glycolic Acid, and 3-Methyl-2-Oxovaleric Acid were identified as crucial factors for diagnosis and potential treatment strategies.

CONCLUSIONS: The data obtained from the study highlights the significant potential of metabolites in assessing infertility and gaining a more in-depth understanding of the underlying pathological mechanisms.

Key Words:
Male infertility, Metabolomics, Organic acids, Azoospermia, Oligospermia.

Introduction

Infertility is defined as the failure to achieve pregnancy after a year of consistent, unprotected sexual intercourse. It is a well-recognized global public health issue and is classified as a medical condition by the World Health Organization, impacting approximately 10-15% of couples aged 15-49[1]. Nearly half of all infertility cases within couples are attributed to male factors. However, a significant proportion of these cases remains unexplained and classified as idiopathic[2]. Male infertility primarily arises from factors like insufficient sperm count, decreased semen volume, low sperm viability, abnormal sperm morphology, and reduced sperm motility[3,4]. The etiology of infertility encompasses sexual, urogenital, congenital, acquired, and various other factors, including varicocele, endocrine disorders, immunological factors, idiopathic semen abnormalities, and other medical conditions[5]. Among these, idiopathic factors constitute the highest proportion[6]. Idiopathic male infertility can be attributed to diverse elements, including chronic stress, endocrine disturbances resulting from environmental pollution, unhealthy dietary habits, radiation, and chemical exposure, urogenital anomalies, metabolic disorders, reactive oxygen species (ROS), and genetic anomalies[7].

Common genetic anomalies prevalent in infertile men involve chromosomal irregularities and

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gene copy number variations. Seminal fluid is primarily produced by the sex glands, including spermatozoa and their precursors[8]. This fluid, rich in diverse molecules, creates the optimal environment for sperm survival and fertilization within the female reproductive system[8]. Consequently, analyzing seminal fluid composition using metabolomic techniques enhances our understanding of the underlying mechanisms of male infertility and aids in discovering novel biomarkers linked to infertility[9]. Recently, genomic, proteomic, and metabolic techniques have gained popularity as research[9] focuses on deciphering the causes of idiopathic infertility, aiming to uncover new diagnostic and therapeutic markers. Metabolomics, as a relatively new concept, has gained prominence in diagnosing various diseases. It involves identifying, quantifying, and characterizing metabolites crucial for intercellular communication found in biological fluids or tumor tissues[9].

Leveraging high-throughput technologies and quantitative analysis methods, metabolomics offers a comprehensive perspective[10]. While genomics and proteomics provide insights into potential outcomes, metabolomics paints a more definitive picture of actual occurrences. The holistic measurement of all metabolites, encompassing acids, carnitines, lipids, and organic compounds, facilitated by metabolomics, presents an ideal approach for disease diagnosis and evaluates the effects of toxic substances on phenotype[10].

Organic acid biomarkers represent metabolic intermediates generated within central pathways of energy production, neurotransmitter breakdown, detoxification, and gut microbial activity[11]. The accumulation of specific organic acids typically signals metabolic deficiencies, which can contribute to fertility issues. These deficiencies might stem from toxicity, nutritional insufficiencies, or hereditary enzyme deficits[12].

As a result, organic acid profiling stands as a significant tool for identifying the root causes of idiopathic infertility, offering comprehensive insights into bodily processes. Advancements in cutting-edge methodologies, including genomics, transcriptomics, proteomics, and metabolomics[13], have led to more precise diagnoses and tailored treatment plans for male infertility. Among these innovative approaches, metabolomics, the most recent technological breakthrough, holds tremendous potential for comprehending and addressing male infertility. Its swift analysis capabilities play an increasingly vital role in biomarker identification for diagnosis[14]. Despite numerous studies on male infertility can be found in the literature, several unanswered questions remain. Bearing this in mind, future research carries the promise of delivering invaluable insights.

Patients and Methods

Selection of Samples

This prospective study was conducted at the In Vitro Fertilization Center and Urology Services of the Department of Obstetrics at Harran University. Sperm samples were collected from three groups: the normospermia group (32 individuals), the oligospermia group (32 individuals), and the azoospermia group (32 individuals). Written informed consent was obtained from all participating individuals, and the study received approval from the Ethics Committee of Harran University Faculty of Medicine. Furthermore, the study adhered to the principles outlined in the Declaration of Helsinki. The Ethics Committee at Harran University approved the study under protocol number 10.01.2023-196576.

Preparation of Seminal Fluid Samples

The patients' sexual abstinence, ranging from a minimum of 2 to a maximum of 5 days, was taken into consideration. We employed sterile plastic sperm collection containers to store the samples. To minimize the post-ejaculation impact on spermatozoa, we incubated the samples at 37°C in an incubator for a specified duration, ranging from 30 to 60 minutes. Following this, the collected semen samples were thoroughly blended in a sterile container using disposable 2.5 ml pipettes to ensure even distribution. Subsequently, the amalgamated samples underwent semen analysis using a microscope.

Evaluation of Sperm Concentration and Motility

A 10 µl semen sample was placed onto the Makler Chamber and examined under a light microscope at 20X magnification. This procedure was repeated a minimum of 3 times, with the results subsequently averaged. Semen concentrations of 15 million sperm per milliliter or higher indicated normospermia. Sperm concentrations below 15 million sperm per milliliter were categorized as oligospermia, while samples devoid of any sperm cells in a milliliter of semen were classified as azoospermia. The collected semen

samples from the patients underwent centrifugation at 5,000 rpm for 10 minutes. This subsequent centrifugation facilitated the isolation of seminal plasma, which was then stored at -20°C.

Organic Acide Profile Analysing with LC-MS/MS

We quantified 54 organic acids using a JASEM (JASEM, Lot No.: CL-550220913, Istanbul, Turkey) Organic Acids Kit, certified for liquid chromatography-mass spectrometry/mass spectrometry (LC/MS-MS). To establish a calibration curve, we utilized six calibration standards supplemented with a stable isotope-labeled organic acid mixture as the internal standard. Mass spectrometric detection was performed in both negative and positive ionization modes (2ESI/+ESI) using multiple-reaction monitoring modes. Chromatographic separations were executed with a JASEM organic acid column set at a column temperature of 40°C, employing a gradient solution flow rate of 0.4 mL/min. In the preparation of the samples, semen samples were diluted tenfold with a JASEM reagent. These diluted samples were then mixed with the internal standard, followed by vortexing and centrifugation. The resulting supernatant was transferred into an HPLC vial for subsequent analysis *via* LC/MS-MS, utilizing the LCMS-8045 machine from Shimadzu, Kyoto, Japan.

Statistical Analysis

The data acquired from the study were analyzed using SPSS software for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). We assessed the normality of the data through the Shapiro-Wilk test. For data following a normal distribution, we employed the mean and standard deviation, while for data lacking a normal distribution, we relied on the median and interquartile range. To analyze parameters with a normal distribution, we applied the paired *t*-test, whereas, for parameters deviating from a normal distribution, we utilized the Wilcoxon test. A significance level of $p<0.05$ was set as the threshold for statistical significance.

Results

Organic Acid Levels Differed Between the Semen Groups

Table I presents demographic information and spermiogram analysis results for individuals with normospermia, oligospermia, and azoospermia. Meanwhile, Table II displays the organic acid profiles of semen fluid from normospermic individuals, as well as seminal fluid from oligospermic and azoospermic individuals. The study's findings reveal significant differences among these groups. When comparing normospermic individuals with those having oligospermia, the latter group exhibited a noteworthy decrease in the levels of 2-OH-Isovaleric Acid, 3-Methyl-2-Oxovaleric Acid, Ethyl-Malonic Acid, Citric Acid, Oxoproline, Malic Acid, N-Acetyl-Aspartic Acid, Suberic Acid, Glutaconic Acid, and Succinic Acid. Similarly, in the comparison between normospermic and azoospermic individuals, a significant reduction was observed in the levels of Citric Acid, Malic Acid, and Suberic Acid within the azoospermia group. Furthermore, when individuals with oligospermia were compared to those with azoospermia, it became evident that the azoospermia group displayed significantly higher levels of 3-Methyl-2-Oxovaleric Acid, Ethyl-Malonic Acid, Glycolic Acid, Glutaconic Acid, and Succinic Acid in contrast to their oligospermic counterparts (*$p<0.05$; **$p<0.01$).

Differences in the Order of Importance of Organic Acid Levels were Observed Among the Semen Groups

Variable Importance in the Projection (VIP) graphs were created to rank the significance of

Table I. Clinical parameters of the patients enrolled in the study.

	Normospermia	Oligospermia	Azoospermia
N	32	32	32
Age	32 ± 3	33 ± 2	34 ± 4
Spermatozoa (Total)	183.3	57.4	-
Motile Spermatozoa (Mean)	94.5	32.2	-
Motility% (Mean ± SD)	46.3 ± 4	31.9±3	-
Progressive Motility% (Mean ± SD)	20.8 ± 2.3	9.4 ±2.6	-
"In situ" Motility (Mean ± SD)	9.4 ± 1.1	10 ± 5.5	-

Table II. Organic acid profiles of normospermia, oligospermia, and azoospermia groups.

Organic acids	Groups			*p*-value	Post Hoc
	Normospermia (A)	Oligospermia (B)	Azoospermia (C)		
2-OH-Isovaleric Acid[x]	0.970 ± 0.263	0.812 ± 0.255	0.862 ± 0.223	0.037[a]	A-B
2-OH-Glutaric Acid[y]	1.583 ± 1.323	0.953 ± 0.658	0.923 ± 0.451	0.078	-
3-OH-Isovaleric Acid[y]	0.142 ± 0.83	0.113 ± 0.065	0.114 ± 0.054	0.102	-
3-OH-Glutaric Acid[y]	0.312 ± 0.174	0.309 ± 0.272	0.378 ± 0.266	0.308	-
3-Methyl-2-Oxovaleric Acid[y]	58.393 ± 41.141	23.234 ± 20.434	45.404 ± 30.577	< 0.001[c]	A-B. B-C
4-Methyl-2-Oxovaleric Acid[y]	59.746 ± 37.915	45.961 ± 42.917	47.375 ± 31.830	0.133	-
4-OH-Phenyl-Lactic Acid[y]	2.085 ± 1.308	1.713 ± 1.386	1.754 ± 0.889	0.340	-
Ethyl-Malonic Acid[y]	0.607 ± 0.365	0.410 ± 0.351	0.816 ± 0.578	0.003[c]	A-B. B-C
Citric Acid[x]	2,456.899 ± 1,326.380	1,697.824 ± 974.765	1,781.394 ± 801.393	0.009b	A-B. A-C
Oxoproline[y]	151.544 ± 115.633	94.372 ± 79.316	119.393 ± 71.515	0.048[c]	A-B
Glycolic Acid[x]	2.684 ± 1.334	2.288 ± 1.446	3.485 ± 1.485	0.004[a]	B-C
Malic Acid[y]	6.502 ± 3.489	4.312 ± 2.832	4.167 ± 2.760	0.004[c]	A-B. A-C
N-Acetyl-Aspartic Acid[y]	0.206 ± 0.137	0.119 ± 0.062	0.182 ± 0.127	0.012[c]	A-B
Suberic Acid[y]	0.182 ± 0.171	0.077 ± 0.046	0.089 ± 0.030	0.012[c]	A-B. A-C
Glutaconic Acid[y]	54.273 ± 42.159	23.131 ± 21.626	36.708 ± 21.834	0.001[c]	A-B. B-C
Pyruvic Acid[x]	74.278 ± 39.955	65.641 ± 39.904	65.964 ± 33.393	0.588	-
Succinic Acid[y]	0.011 ± 0.008	0.007 ± 0.005	0.011 ± 0.007	0.026[c]	A-B. B-C

The data for continuous variables were presented as mean ± standard deviation. Group comparisons were performed using either the One-Way ANOVA or Kruskal-Wallis tests, with a significance level of $p < 0.05$. In cases where variables showed statistical significance, post hoc pairwise group comparisons were conducted using the Tukey, Games Howell, or Mann-Whitney tests. [x]One-Way ANOVA Test; [y]Kruskal-Wallis Test; [a]Tukey Test; [b]Games Howell; [c]Mann-Whitney Test.

differences in organic acid results among the oligospermia, azoospermia, and normospermia groups (Figure 1). A high VIP score indicates a greater contribution to the differentiation between these groups. The three organic acids with the highest VIP scores were Ethyl-Malonic Acid, Glycolic Acid, and 3-Methyl-2-Oxovaleric Acid, respectively. These organic acids play a pivotal role in effectively distinguishing between the groups.

The Heatmap Illustrates Variations in Organic Acid Levels between the Groups

A heatmap was generated to visually illustrate the concentrations of the analyzed organic acids within the groups (Figure 2). An increase in concentration is depicted by brown coloring, while blue represents a decrease. Additionally, the depth of the brown or blue shading indicates the extent of the increase or decrease in organic acid levels. Upon reviewing the heatmap, a significant decrease was observed in the concentrations of almost all analyzed organic acids within the oligospermia and azoospermia groups when compared to the normospermia group. Furthermore, the group of oligospermia exhibited a decrease in most organic acid levels compared to the group of azoospermia.

Discussion

A male factor contributes to infertility in 50% of couples. Unfortunately, the cause of male factor infertility remains idiopathic in nearly 50% of cases. While semen analysis (SA) serves as the initial diagnostic step for male infertility, it is hindered by considerable sample variability[15]. Given the limited availability of diagnostic tools, there is a clear need for minimally invasive diagnostics to gain a deeper understanding of the etiology of male factor infertility[15]. Genetic factors play a significant role in male infertility, with numerous potentially involved genes identified[16]. The objective of this study was to investigate variations in organic acid levels among male infertility groups. We analyzed seminal fluid samples from 96 patients, categorizing them into three groups: normospermia (32), oligospermia (32), and azoospermia (32). All participants were recruited from the infertility clinic at Harran University Medical Faculty Hospital. The study aims to contribute to the establishment of biomarkers in diagnosing the causes of male infertility by exploring the roles of implicated genes, epigenetic modifications, proteins, and metabolites.

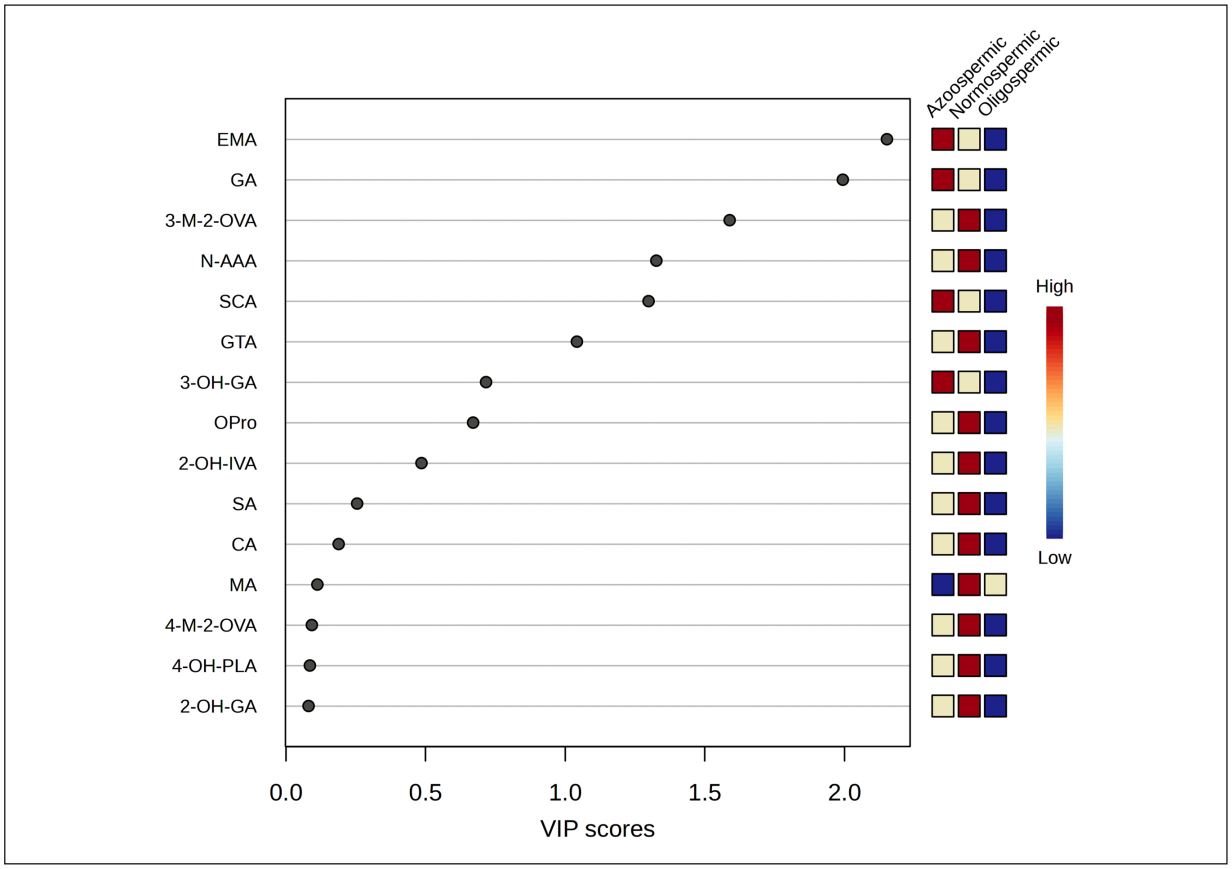

Figure 1. VIP plot: important organic acids identified in order of increasing importance (VIP: Variable Importance in the Projection).

The extensive exploration of LC-MS/MS in studying the male reproductive system has been limited, rendering it an underutilized resource in the pursuit of male infertility biomarkers[17]. This study aims to delve further into this field, evaluating its potential in aiding the diagnosis of male infertility. Despite the introduction of the concept of the "metabolome" over two decades ago, there remains a lack of studies in the domain of male infertility. Hence, this study represents a significant contribution to the field. In recent years, research[18] on the sperm metabolome has grown, encompassing both humans and agricultural animals. Substrates, products, and byproducts from anabolic and catabolic reactions, critical in sperm processes, could potentially serve as markers for sperm metabolic health[19]. Consequently, the science of andrology shows a keen interest in employing metabolomics as a promising avenue for discovering indicators of sperm quality and fertilizing capacity[20].

Male infertility emerges from a blend of genetic and epigenetic factors, constituting a complex issue[21]. This intricate reality underscores the unexplained (idiopathic) cause of a substantial portion of male infertility[22]. Epigenetic changes present a potential explanation for idiopathic male infertility[23,24]. In their study, Wu et al[25] demonstrated that unexplained male infertility is linked to hypermethylation of sperm DNA within the *MTHFR* gene promoter. They observed a significant increase in *MTHFR* gene promoter hypermethylation solely in the oligoasthenoteratozoospermic group, referred to as patient subgroup-b. Still, this increase was not observed in normozoospermic men, labeled as subgroup-a. As previously mentioned, oxidative stress can induce epigenetic alterations in sperm and generate ROS. Infertile males often exhibit elevated ROS levels, which correlate with changes in sperm shape, concentration, motility, and increased DNA fragmentation[26].

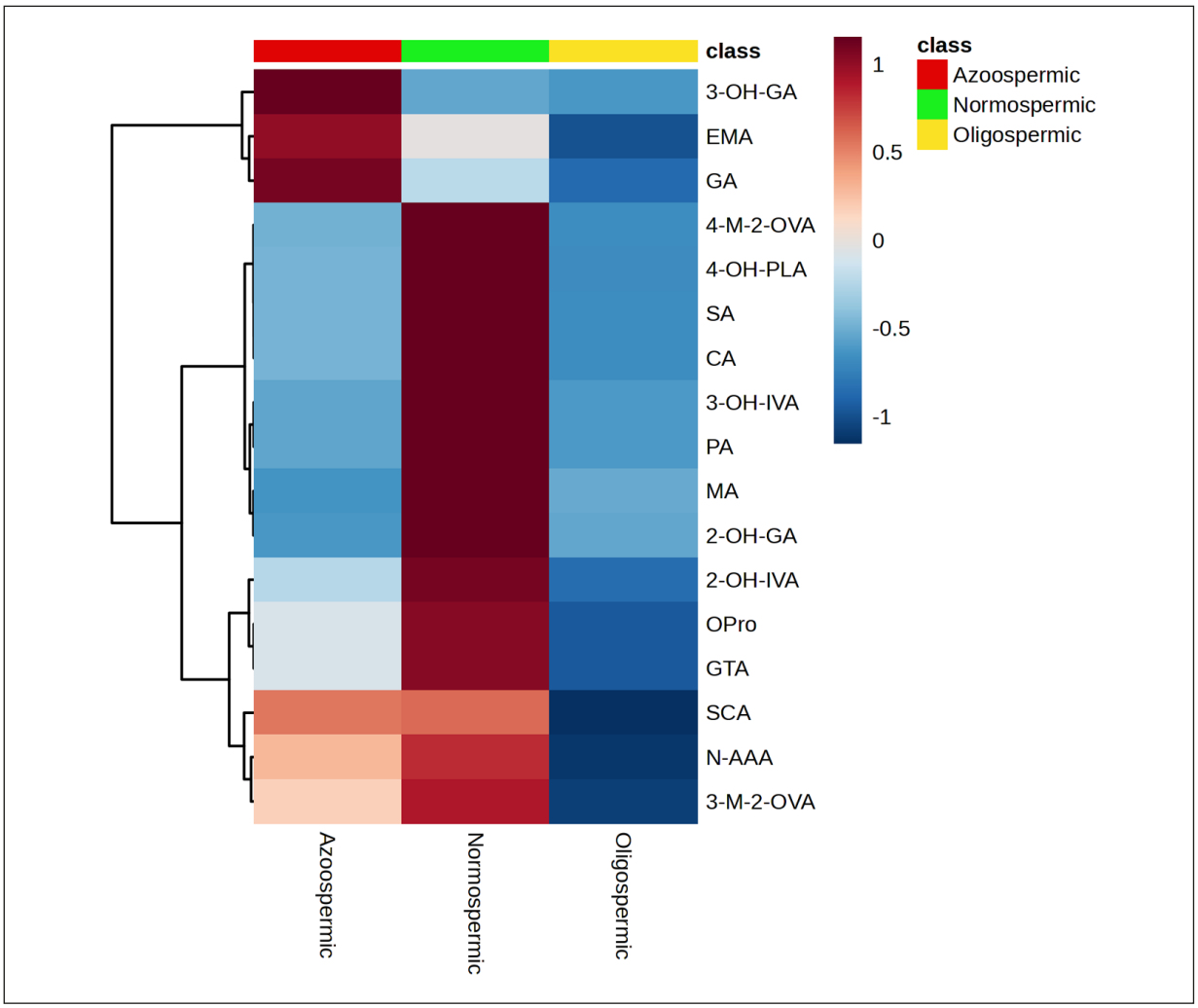

Figure 2. Heat map showing the concentrations of organic acids in the groups.

In the research by Gilany et al[27], Raman spectroscopy was employed to assess ROS levels in the ejaculation of 20 azoospermic patients for spermatogenesis evaluation. The outcomes enabled the differentiation of patients with spermatogenesis from those without, further classifying patients with negative testicular sperm extraction into subcategories like hypospermatogenesis, sperm maturation arrest, and Sertoli-only syndrome. Another study by Gilany et al[28] examined the seminal plasma of azoospermic men undergoing testicular sperm extraction. This investigation utilized untargeted metabolomics profiling through gas chromatography-mass spectrometry (GC-MS) and successfully differentiated between azoospermic male sperm, identified during testicular sperm extraction. In a separate study, Zhao et al[29] employed GC-MS to explore sperm metabolites associated with idiopathic asthenozoospermia and identified several amino acids, energy, and nucleoside metabolites with altered Levels. Qiao et al[30] also utilized GC-MS to analyze seminal plasma, revealing 44 differentially expressed metabolites in individuals with unexplained infertility and normal semen analysis compared to fertile controls. These metabolites primarily pertained to amino acid metabolism changes. The researchers[30] proposed their metabolite profiling platform as a diagnostic tool for idiopathic infertility, achieving an 82% differentiation rate for infertile patients.

Similarly, in a meta-analysis, Guo et al[31] found that males with normal body mass index (BMI) and semen parameters significantly differed from obese men with abnormal semen parameters in terms of 16 metabolites identified in seminal

plasma. Specifically, the metabolism of arginine, proline, beta-alanine, and glutathione exhibited significant alterations, and notably higher levels of spermidine and spermine were observed in obese males with abnormal semen parameters[31]. The potential application of proteomic and metabolomic profiling for diagnosing male infertility remains promising due to its ability to distinguish infertility phenotypes with minimal invasiveness[32]. However, the widespread clinical utilization of these profiling panels is currently restricted, primarily due to the extensive range of proteins, peptides, and metabolites present in semen samples, along with their susceptibility to variation caused by environmental factors among individuals. Further research is imperative before implementing these profiling panels on a larger scale[33].

In the study, based on VIP score analysis, the three organic acids with the highest scores were Ethyl-Malonic Acid, Glycolic Acid, and 3-Methyl-2-Oxovaleric Acid. A notably high amount of Ethyl-Malonic Acid was observed in individuals with azoospermia. Malonic acid's presence has been shown[34] to exhibit a spermicidal effect, even with a mere 0.1% addition. It can restrict the use of carbohydrates as an energy source in sperm by inhibiting intermediate substrates in the Krebs cycle. A prior study[35] discovered that malonate inhibits the respiration and motility of sperm in the absence of glucose, implying that heightened levels of malonic acid in the seminal plasma of individuals with azoospermia might lead to sperm death[36]. Among the key parameters influencing male fertility in mammals are sperm (spermatozoa) motility and progression[37]. Sperm motility can be impaired by various drug usages and environmental factors. Decreases in sperm motility and progression have been demonstrated[38] to be a direct cause of male infertility in diverse mammalian species. Variations in sperm motility and progression values display the strongest direct correlation with infertility[39]. Hence, studies focusing on enhancing sperm motility should be conducted.

In a patented study[40], glycolic acid was examined, and the findings suggested that it enhances sperm motility and could potentially help alleviate infertility. Glycolic acid (GA) is a compound with the IUPAC name 2-hydroxyethanoic acid and the molecular formula $C_2H_4O_3$. Glycolic acid is also present in sugar beets, sugar cane, and various fruits. It is utilized as a flavoring agent, a food processing preservative, and a skincare component in the pharmaceutical industry. In the wake of a patent study[40], the impact of glycolic acid on mouse and bull sperm motility and progression at various time intervals post-administration was assessed. Experimental outcomes revealed that glycolic acid significantly increased sperm motility and progression shortly after the administration when compared to untreated control sperm.

In our study, we determined that the glycolic acid level was lower in individuals with oligospermia compared to those with normospermia, whereas it was found to be elevated in individuals with azoospermia. The metabolite 3-Methyl-2-oxovaleric acid is generated from the incomplete breakdown of branched-chain amino acids and belongs to the category of keto-acids, a type of organic acid. Elevated levels of organic acids can lead to metabolic acidosis, evidenced by a decrease in arterial pH below 7.35. Due to its acidic properties, 3-Methyl-2-oxovaleric acid is considered detrimental, causing acidosis and exerting adverse effects on various organ systems. Interestingly, contrary to expectations, research in the literature has revealed its higher presence in the seminal fluid of individuals with normospermia. However, individuals with azoospermia and oligospermia exhibit lower levels of this metabolite, potentially contributing to a decline in seminal fluid pH and consequently a reduction in sperm count.

Conclusions

The use of metabolomics as a tool in male infertility research represents a novel and promising advancement, with the potential to reveal biomarkers and elucidate their underlying causes. Despite its promise, this approach has not yet gained widespread adoption in clinical settings for diagnosing male infertility. In other words, this approach has not been widely embraced. Our study identified specific organic acids, including Ethyl-Malonic Acid, Glycolic Acid, and 3-Methyl-2-Oxovaleric Acid, which ranked highest in the VIP score analysis. These findings suggest significant potential for diagnosing and treating male infertility. Our study contributes to the growing body of research on this subject, laying the groundwork for future, more comprehensive molecular investigations aimed at enhancing prevention and treatment strategies for infertility.

Conflict of Interest
The authors declare that they have no conflict of interests.

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Ethics Approval
This study was approved by the Harran University Ethics Committee (protocol number 10.01.2023-196576).

Informed Consent
All patients signed informed consent before the procedure.

Authors' Contribution
E.A., I.K and H.U. designed the study. E.A., I.K., E.T. and M.A. did the literature search. E.A., I.K., E.T. M.R. and H.U. collected the data. E.A., I.K., E.T. M.R. and H.U. analyzed and interpreted the data and wrote the manuscript.

Data Availability
The data that support the findings of this study are available from the corresponding author, [E.A.], upon reasonable request.

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