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Comparison of sperm carnitine profiles of normospermic, oligospermic and azospermic individuals

E. ARSLAN[1], I. KOYUNCU[2], E. TEMIZ[3], M. ARI[4], S. AKMESE[2], M. RAMAT[5]

[1]Department of Urology, Faculty of Medicine, Usak University, Usak, Turkey
[2]Department of Medical Biochemistry, Faculty of Medicine, Harran University, Sanliurfa, Turkey
[3]Program of Medical Promotion and Marketing, Health Services Vocational School, Harran University, Sanliurfa, Turkey
[4]Program of Home Patient Care, Söke Health Services Vocational School, Aydin Adnan Menderes University, Aydin, Turkey
[5]Program of Orthopedic Prosthesis and Orthetics, Health Services Vocational School, Harran University, Sanliurfa, Turkey

Abstract. – OBJECTIVE: The World Health Organization recognizes infertility as a public health issue. An estimated 48.5 million couples worldwide grapple with infertility. Infertility and spermatogenesis dysfunction arise from diverse genetic factors, including single-gene mutations and chromosomal abnormalities. Current research continues to explore other potential causes of infertility, leveraging both proteomic and metabolomic analyses. The primary aim of this study is to underscore the significance of investigating male infertility from a metabolomic perspective.

PATIENTS AND METHODS: This study aimed to identify new markers for diagnosing and treating male infertility by examining the carnitine profiles in seminal fluids of individuals diagnosed with the normospermic group, oligospermic group, and azospermic group, employing the LC/MS-MS method.

RESULTS: The first three L-carnitines C2 (Acetylcarnitine), C8:1 (Octenoylcarnitine), and C16:1 (Palmitoleylcarnitine), emerged as potential novel markers for diagnosis and treatment.

CONCLUSIONS: Based on these findings, we posit that the results obtained in this study will aid in diagnosing, treating, and monitoring systemic diseases, and provide a foundation for more comprehensive future molecular studies aimed at enhancing prevention and treatment strategies for infertility.

Key Words:
Normospermic, Oligospermic, Azospermic, Carnitine, Metabolomics.

Introduction

Infertility is recognized as a social health problem by the World Health Organization. It is estimated that there are approximately 48.5 million couples worldwide experiencing infertility. A variety of genetic factors, such as single gene mutations and chromosomal abnormalities, are known to cause failures in spermatogenesis and sperm deterioration. However, the influence of other factors on infertility is currently under investigation through proteomic and metabolomic analyses[1]. Despite male factors accounting for a significant proportion of infertility cases, female factors can also be contributory. Male infertility is primarily attributed to a deficient sperm count resulting from inadequate sperm concentration, diminished total semen volume, low sperm viability, abnormal sperm morphology, and poor sperm motility[2]. Etiological causes of infertility encompass sexual factors, urogenital infections, congenital anomalies, acquired factors, varicocele, endocrine disorders, immunological factors, idiopathic semen disorders, and other diseases[3]. L-carnitine is one of the recommended treatments for male infertility[4].

L-carnitine, a cofactor in the transport and subsequent oxidation of long-chain fatty acids within mitochondria, primarily provides an energetic substrate in the epididymis[5]. Synthesized from amino acids, L-carnitine is marketed as a nutritional supplement and an ergogenic source of physical strength aimed at improving the vitality of living organisms. Consequently, it has been used to boost physical performance as a dietary supplement and has spurred reasearch. However, the various effects of L-carnitine supplementation on proteomic and metabolomic profiles are yet to be fully understood[6]. Therefore, future research efforts in this direction hold significant importance.

Corresponding Author: Erkan Arslan, MD; e-mail: drurology.erkan@gmail.com

Currently, genomics, proteomics, and metabolomics have emerged as popular areas of study in the quest to understand idiopathic causes of infertility. These techniques are employed to develop novel diagnostic and therapeutic markers. Among them, metabolomics – a relatively new concept – has been employed extensively in the investigation and diagnosis of various diseases with unclear etiology[7]. While genomics and proteomics can suggest potential outcomes, metabolomics provides a snapshot of "what has actually transpired". Consequently, detailed and quantitative measurement of all metabolites (a metabolomic approach) holds an advantage for disease diagnosis and assessing the impacts of toxic agents on phenotype[8]. Metabolomic analyses can be performed on various body fluids such as serum, urine, cerebrospinal fluid, seminal fluid, plasma, and saliva, finding use in clinical biochemistry, pharmacology, preclinical drug trials, toxicology, transplant monitoring, cancer metabolism, and newborn screening[9]. Despite extensive research on the diagnosis and treatment of male infertility, numerous questions remain unanswered[10].

This study seeks to elucidate the role of seminal fluid carnitine in the diagnosis and treatment of male infertility. The data derived may introduce innovative diagnostic and therapeutic strategies for male infertility. This exploration of the carnitine profile in seminal fluids of individuals grappling with male infertility – a burgeoning problem – could prove invaluable in identifying new diagnostic markers and treatments. We anticipate that the changes in profiles discovered through the study will yield disease-specific findings, shedding light on the lingering questions in this field.

Patients and Methods

Samples Selection

This prospective study was conducted at the *in vitro* fertilization center and urology services of Harran University, Department of Obstetrics. The study collected sperm samples from 30 men, each with normospermic group, oligospermic group, and azospermic group. Written informed consent was obtained from all participants, and the study received approval from the Harran University Faculty of Medicine's Ethics Committee. Furthermore, the study adhered to the Declaration of Helsinki. The Ethics Committee at Harran University approved the study (protocol number 24.01.2023-200931).

Seminal Fluid Sample Preparation

Participants were instructed to observe sexual abstinence for a minimum of 2 and a maximum of 5 days. Sperm samples were collected in plastic sterile sperm collection containers. To avoid negatively impacting the spermatozoa post-ejaculation, the samples were incubated at 37°C for a survival time of 30 to 60 minutes. The resultant samples were mixed evenly in a sterile container using disposable 2.5 ml pipettes. Once distribution was completed, the samples underwent semen analysis under a microscope. Evaluation of Sperm Concentration and Motility involved placing 10 µl of the semen sample on a Makler Chamber and assessing it under a light microscope at 20x magnification. This process was performed at least three times, and results were evaluated by averaging the numbers. A sperm concentration of 15 million or more per millimeter of semen was classified as normospermic group. A sperm concentration of fewer than 15 million sperm per millimeter of semen was classified as oligospermic group, while samples devoid of sperm cells in a millimeter of semen were classified as azospermic group. Semen samples collected from patients were centrifuged at 5,000 rpm for 10 minutes. The seminal plasma, obtained through centrifugation, was stored at -20°C.

L-carnitine Profile Analysing with LC-MS/MS

The carnitine profile was evaluated by modifying the neonatal screening method developed by La Marca et al[11]. We applied 100 µl of semen samples onto Guthrie papers and let them dry at room temperature. We then cut the Guthrie papers into 3.2 mm disks and placed them in 96-well plates. The spot sample was extracted and butylated by dispensing 300 µl of an extraction solution consisting of methanol and aqueous solution of 3 mmol/L hydrate hydrazine at 37°C for 25 min. For internal standards, we used stable heavy isotope analogs of carnitine and acylcarnitine [Labeled Carnitine Standards Set B (Cambridge Isotope Laboratories)] in the extracted solution. The extracted samples was injected into the LCMS-8040 device (Shimadzu Corporation, Japan). All collected data were reprocessed using Shimadzu Neonatal Software, which automatically calculates the concentration of each component.

Statistical Analysis

We performed statistical analyses of the data using IBM Statistical Package for the Social Sciences (SPSS) v25 (IBM Corp., Armonk, NY, USA). We evaluated descriptive statistics (body weight, age, height, gender, BMI), including arithmetic mean ($\bar{X}$), standard deviation (SD), and minimum and maximum value statistics. We used the Kolmogorov-Smirnov test to evaluate whether the data showed normal distribution. Mean±standard deviation analysis was used for normally distributed data. We used an Independent-sample *t*-test to compare numerical values between the two groups. We analyzed relationships between variables with the Pearson and Spearman Correlation coefficients. A $p<0.05$ was considered statistically significant. Variable Importance in the Projection (VIP) score and heatmap were generated with MetaboAnalyst 5.0 program.

Results

L-carnitine Levels Differed between the Groups

Table I presents the demographic information and semen analysis results of individuals with normospermic group type (group 1), oligospermic group (group 2), and azospermic group (group 3) types. Table II presents the L-carnitine profile results of seminal fluid from individuals with normal sperm, oligospermic group, and azospermic group. A comparison of individuals with the normospermic group and the oligospermic group revealed levels of Acetylcarnitine, Hexanoylcarnitine, Octenoylcarnitine, and Palmitoylcarnitine and decreased levels of Methylmalonylcarnitine. Furthermore, when comparing individuals with the Oligospermic group type to those with the Azospermic group type, we observed elevated levels of Acetylcarnitine, Tiglylcarnitine, Hexanoylcarnitine, Octenoylcarnitine, and Palmitoleylcarnitine (*$p<0.05$; **$p<0.01$).

Differences were Found in the Order of Importance of L-carnitines between Groups

Variable Importance in the Projection (VIP) graphs were generated to rank the differences among the normospermic group, oligospermic group, and azospermic group in order of importance according to the L-carnitine results (Figure 1). A high VIP score suggests an increased contribution to the separation between the groups. The three L-carnitines with the highest VIP scores were C8:1, C2, and C16:1, respectively.

L-carnitine Levels Differed between Groups in the Heat-map.

We constructed a heat map to visualize the concentrations of the L-carnitines analyzed in the groups (Figure 2). In this representation, increased L-carnitine concentration is shown in brown and decreased concentration in blue. Additionally, the depth of brown and blue signifies the extent of the increase or decrease in L-carnitine levels. Upon examination of the heat map, we found that all the L-carnitine concentrations we analyzed significantly decreased in the Oligospermic group and normospermic group compared to the azospermic group. Furthermore, the decrease in the normospermic group and oligospermic group was significantly higher than in the azospermic group.

Discussion

Male factor infertility is implicated in 50% of couples struggling with fertility issues. Regrettably, in approximately 50% of these cases, the cause remains idiopathic. While semen analysis remains the initial diagnostic step for male infertility, it is an imperfect test with substantial sample variability. Given the paucity of diagnostic tools, there is a pressing need for minimally invasive diagnostic methods that can improve our understanding of the causes of male infertility.

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

	Normospermic	Oligospermic	Azospermic
N	30	30	30
Age	35.1±5	37.3±7	35.9±6
Total Spermatozoa (10^6)	183.3	57.4	-
Motile Spermatozoa (10^6) (mean)	95.3	30.4	-
Motility % (mean±SD)	49.3±5	28.9±7	-
Progressive Motility % (mean±SD)	19.5±4.3	8.4±5.6	-
% of "*in situ*" motility (mean±SD)	8.4±2.1	11±6.5	-

Table II. Comparative profiles of L-carnitine in normospermic, oligospermic, and azospermic groups.

Carnitines	Abbreviation	Groups			*p*-value	Post-Hoc
		Normospermic (A)	Oligospermic (B)	Azospermic (C)		
Free Carnitine[x]	C0	30.180±8.510	29.409±7.950	27.612±6.777	0.403	-
Acetylcarnitine[x]	C2	7.022±3.228	6.654±3.299	10.050±4.006	0.001[a]	A-C. B-C
Propionylcarnitine[x]	C3	1.228±0.498	1.351±0.676	1.671±0.960	0.051	-
Butyrylcarnitine[x]	C4	0.166±0.056	0.161±0.072	0.175±0.061	0.686	-
Methylmalonylcarnitine[x]	C4DC	0.322±0.115	0.286±0.111	0.244±0.066	0.009[b]	A-C
Isovalerylcarnitine[x]	C5	0.218±0.058	0.201±0.063	0.214±0.051	0.463	-
Tiglylcarnitine[x]	C5:1	0.208±0.056	0.193±0.046	0.232±0.064	0.025[a]	B-C
Hydroxyisovalerylcarnitine[x]	C5-OH	0.417±0.106	0.380±0.101	0.369±0.127	0.201	-
Glutarylcarnitine[x]	C5DC	0.079±0.025	0.095±0.030	0.086±0.025	0.055	-
Hexanoylcarnitine[x]	C6	0.041±0.016	0.043±0.016	0.055±0.022	0.006[a]	A-C. B-C
Adipoylcarnitine[x]	C6DC	0.075±0.019	0.076±0.017	0.079±0.013	0.623	-
Octanoylcarnitine[y]	C8	0.053±0.031	0.057±0.030	0.063±0.043	0.579	
Octenoylcarnitine[y]	C8:1	0.241±0.140	0.219±0.100	0.448±0.260	0.029[c]	A-C. B-C
Suberoylcarnitine[x]	C8DC	0.065±0.019	0.063±0.012	0.060±0.013	0.371	-
Decanoylcarnitine[y]	C10[x]	0.055±0.024	0.069±0.035	0.072±0.040	0.194	-
Decenoylcarnitine[y]	C10:1	0.331±0.119	0.365±0.123	0.403±0.165	0.143	-
Sebacoylcarnitine[x]	C10DC	0.038±0.008	0.041±0.014	0.045±0.013	0.069	-
Dodecanoylcarnitine[y]	C12	0.164±0.082	0.161±0.071	0.180±0.086	0.615	-
Myristoylcarnitine[x]	C14	0.139±0.056	0.133±0.051	0.155±0.048	0.228	-
Myristoleylcarnitine[y]	C14:1	0.068±0.027	0.073±0.029	0.078±0.031	0.304	-
Tetradecadienoylcarnitine[y]	C14:2	0.157±0.073	0.156±0.063	0.183±0.070	0.169	-
Palmitoylcarnitine[x]	C16	1.123±0.281	1.164±0.427	1.353±0.434	0.045[b]	A-C
Palmitoleylcarnitine[x]	C16:1	0.175±0.069	0.158±0.070	0.214±0.102	0.021[b]	B-C
Stearoylcarnitine[x]	C18	0.712±0.179	0.725±0.236	0.702±0.146	0.891	-
Oleylcarnitine[x]	C18:1	2.415±0.643	2.619±0.848	2.509±0.574	0.509	-
Linoleylcarnitine[x]	C18:2	0.910±0.300	0.903±0.285	1.048±0.291	0.086	-
Hydroxyoleylcarnitine[x]	C18:1-OH	0.018±0.005	0.020±0.007	0.018±0.004	0.388	-

Data for continuous variables were presented as mean ± SD. One-way ANOVA or Kruskal-Wallis tests were employed for group comparisons. A $p<0.05$ was considered statistically significant. Pairwise group comparisons (Post-Hoc) for variables that showed statistical significance were performed using 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. *Outliers and extreme values were removed from the dataset and replaced with mean values.

It is well-documented[12] that male infertility is primarily influenced by genetic factors, and many genes that could cause infertility were identified. This study aims to examine L-carnitine levels in the seminal fluid of individuals with the Azospermic group type, Oligospermic group, and normospermic group. By using the LC-MS/MS method, a powerful tool in determining carnitine profiles, we aimed to identify potential new markers that could assist in the diagnosis and treatment of idiopathic male infertility. In the current literature, carnitine determination is predominantly used for analyzing blood plasma and urine[13,14]. However, our study ascertains carnitine levels from seminal fluid. Considering the still elusive causes of infertility, we believe the L-carnitine profiles from this study could shed light on the underlying causes of infertility. Simultaneously, we suggest that these findings could provide clinicians with a fresh perspective, serving as a supplementary laboratory test when evaluating male fertility.

In this study, we collected seminal fluid samples from a total of 90 male patients – 30 each with the normospermic group, oligospermic group, and azospermic group, respectively – who sought treatment at the infertility clinic of Harran University Medical Faculty Hospital. We aimed to delineate the differences in carnitine profiles across these groups. Moreover, by using LC-MS/MS, an effective and powerful technique, on se-

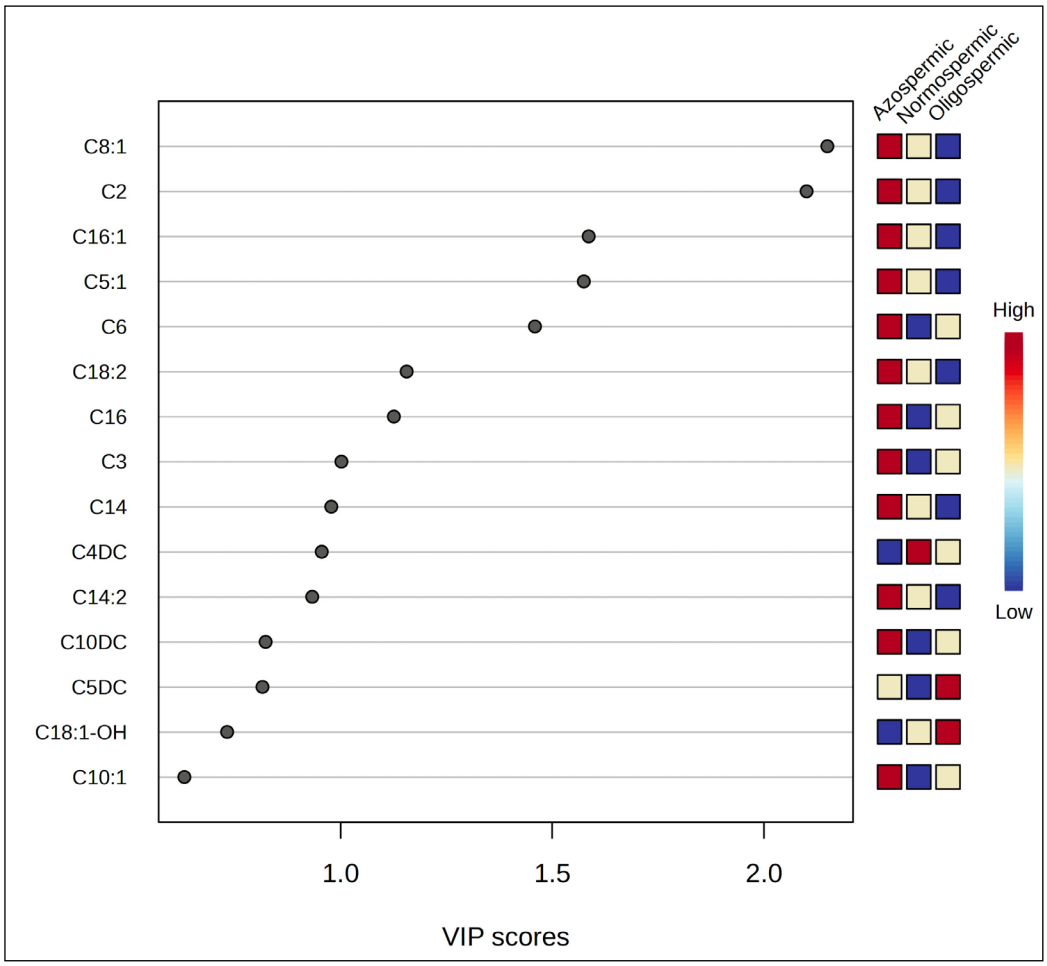

Figure 1. VIP plot: identification of significant L-carnitines in ascending order of importance.

men samples from individuals diagnosed with the normospermic group, oligospermic group, and azospermic group, we also sought to identify new markers that might aid in the diagnosis and treatment of male infertility.

Looking at the literature, it appears that one of the previously employed metabolomics techniques to investigate the male reproductive system is the LC-MS technique. This technique facilitated the identification of several metabolites from human seminal plasma[15]. To the best of our knowledge, even though the term "metabolom" was coined almost 20 years ago, research is urgently needed in the field of male infertility. In this regard, we believe that our study is both valuable and original. In recent years, research into the sperm metabolome in humans and farm animals has increased. Consequently, the science of andrology is taking a keen interest in the application of metabolomics as a promising method for discovering indicators of sperm quality and fertilization capacity[16]. L-carnitine is a compound with potent antioxidant properties, and it specifically inhibits lipid peroxidation. It is recognized for its potential benefits in preventing chronic diseases caused by oxidative stress, owing to its antioxidant properties[17]. Given L-carnitine's significant role in energy metabolism and its various benefits, it has been increasingly used as an alternative treatment agent in human health studies[18-22] in recent years. The compound has found applications in areas such as delaying aging[18], improving memory (notably in preventing diseases like Alzheimer's and Parkinson's)[19], forestalling heart attacks and other heart diseases[20], managing peripheral vascular diseases[21], and chronic kidney failure[22], treating nervous disorders and depression[23], supporting balanced nutrition, dieting, obesity studies and diabetes treatment[24], enhancing sperm maturity and motility[25], and promoting athletes' health[26] serving as both a functional and therapeutic support preparation.

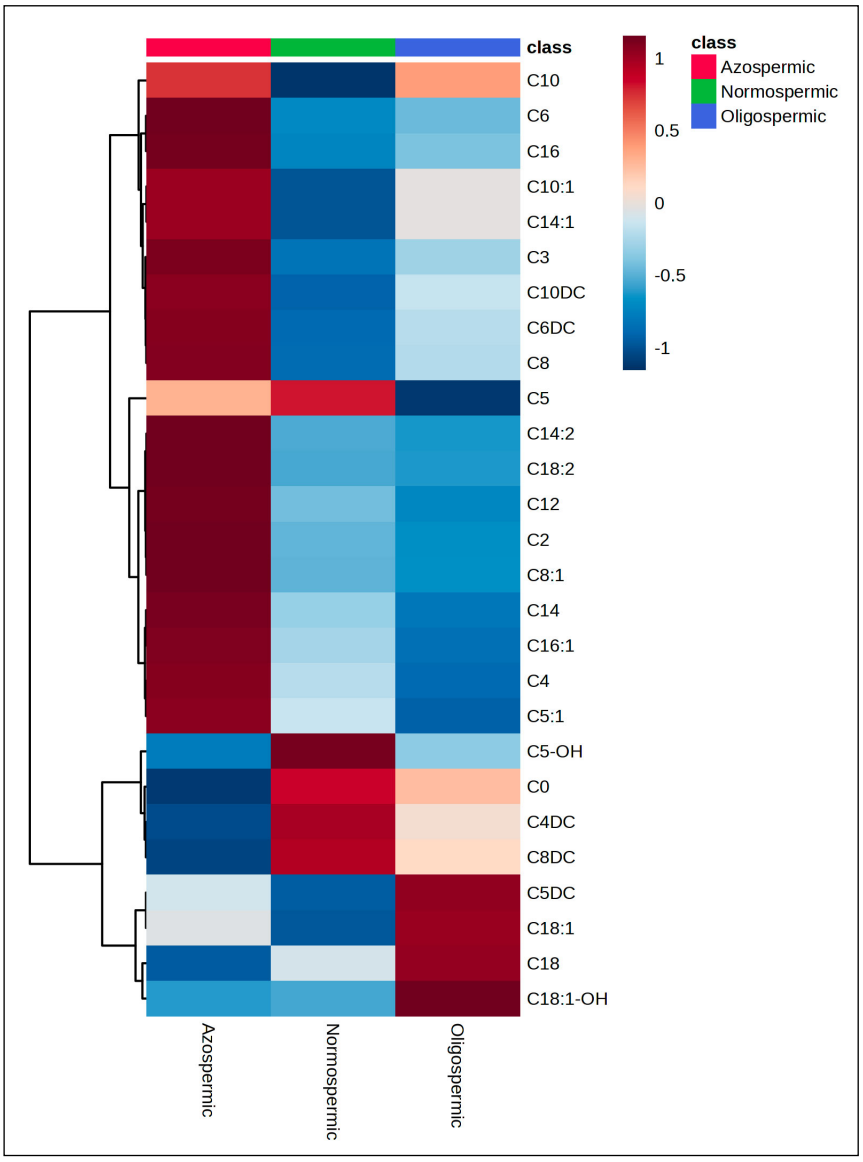

Figure 2. Heat map: representation of L-carnitine concentrations across the groups.

Wang et al[27] demonstrated in their study that L-carnitine can enhance ejaculatory sperm motility in men with asthenozoospermia. In another study, Shi et al[28] revealed that *in vitro* exposure to L-carnitine improved testicular sperm motility. Aliabadi et al[29] reported that L-carnitine, in an *in vitro* setting, can elevate testicular sperm motility of experimental animals. It has been noted in various studies that carnitine levels are low in the sperm of men diagnosed with oligoasthenoteratozoospermia[30]. The results from our study align with these findings. Abdullah et al[31] observed that L-carnitine treatment mitigated the adverse effects of decreased sperm motility and vitality due to inflammation and oxidative stress. L-carnitine bolsters sperm motility by influencing fatty acid metabolism and regulating the quantity of acetyl-CoA[32,33]. Chaipayungpan et al[34], in their study, posited that Acetyl-CoA is essential for the tricarboxylic acid cycle and energy production, hence the augmentation of sperm motility by L-carnitine may be attributed to the effects of L-carnitine on oxidative phosphorylation and energy production. Oxidative stress, which results in functional impairment in the nucleus and mitochondrial pathways of male reproductive cells, inflicts damage to spermatozoa. Reactive oxygen species (ROS), known to play a critical role in the pathophysiology of sperm, is among the primary factors causing male infertility, with infertile men's seminal fluids containing higher levels of ROS than those of fertile men. Seminal plasma

is abundant in Superoxide Dismutase (SOD) and catalase (CAT) enzymes, and these enzymes coexist with antioxidants such as tocopherol, ascorbic acid, and carnitine[35]. Lenzi et al[36] noted that L-carnitine exhibits a positive antioxidant and radical scavenging effect in the treatment of male infertility caused by oxidative stress. Oxidative stress is believed to significantly impact male infertility. Literature focusing on the effect of L-carnitine on key sperm parameters reported a positive correlation between L-carnitine and semen parameters such as sperm count, motility, and morphology[37-40].

In our study, we observed a significant decrease in L-carnitine concentrations in the oligospermic group and normospermic group when compared with the azospermic group. Additionally, the decline in the Normosperm and Oligospermic groups was significantly more pronounced than that in the Azospermic group.

Conclusions

In this LC-MS/MS-based investigation, the sperm carnitine profile of azospermic group and oligospermic group patients was compared with that of normospermic group individuals, highlighting the relevance of carnitine metabolomics. In contexts where sperm analysis may not be feasible, this study potentially equips clinicians with an additional lens for assessing fertility. The utilization of ejaculate analysis, spermiogram parameters, and ejaculate carnitine profiling have augmented our knowledge base, potentially aiding in the diagnosis, management, and monitoring of systemic diseases. Our VIP score analysis points towards the potential diagnostic and therapeutic relevance of certain L-carnitine metabolites, such as C2 (Acetylcarnitine), C8:1 (Octenoylcarnitine), and C16:1 (Palmitoleylcarnitine). Therefore, it may be hypothesized that these findings could lay a foundation for subsequent, more nuanced molecular studies targeting enhanced prevention and management strategies for infertility. However, the strength of these conclusions must be weighed against the constraints and limitations of the present study.

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Ethics Approval
This study was approved by the Harran University Ethics Committee (protocol number 24.01.2023-200931).

Informed Consent
All patients signed informed consent before the procedure.

Conflict of Interest
The authors have no conflicts of interest to declare.

Authors' Contributions
E.A. and I.K. designed the study. E.A., I.K., E.T., and M.A. did the literature search. E.A., I.K., S.A., and M.R. collected the data. E.A., I.K., E.T., M.A., S.A., and M.R. 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.

ORCID ID
E.A.: 0000-0002-3262-2512
I.K.: 0000-0002-9469-4757
E.T.: 0000-0001-8911-7763
M.A.: 0000-0002-1504-7050
S.A.: 0000-0003-4992-0281
M.R.: 0000-0003-4922-6708

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