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SEMINAL-PLASMA FERROPTOSIS MARKERS (GPX4 AND ACSL4) AND THEIR RELATION TO SPERM DNA FRAGMENTATION IN ASTHENOZOOSPERMIC IRAQI MEN

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ABSTRACT

Background: Ferroptosis is an iron-dependent cell death driven by peroxidation of membrane phospholipids. Sperm membranes are rich in polyunsaturated lipids, which makes them a likely target. Glutathione peroxidase 4 (GPX4) blocks this process and acyl-CoA synthetase long-chain family member 4 (ACSL4) feeds it. Neither has been studied in the seminal plasma of Iraqi men. **Methods:** This case-control study enrolled 60 men with isolated asthenozoospermia (progressive motility < 30%) and 60 normozoospermic controls at the Fertility Centre, Al-Diwaniyah, Iraq. Semen was examined by the WHO 2021 manual, seminal-plasma GPX4 and ACSL4 were measured by sandwich ELISA, and the sperm DNA fragmentation index (DFI) was read with the sperm chromatin dispersion test. **Results:** Compared with controls, asthenozoospermic men had significantly lower GPX4 (5.18 ± 1.76 vs 8.52 ± 2.34 ng/mL; $p < 0.001$) and significantly higher ACSL4 (6.37 ± 1.88 vs 3.84 ± 1.25 ng/mL; $p < 0.001$). The ACSL4/GPX4 ratio was 1.36 ± 0.51 vs 0.48 ± 0.20 ($p < 0.001$), and DFI was $32.7 \pm 10.4\%$ vs $15.3 \pm 5.8\%$ ($p < 0.001$). Within the case group, DFI correlated with GPX4 ($r = -0.44$; Bonferroni $p = 0.0008$) and with ACSL4 ($r = 0.41$; Bonferroni $p = 0.002$). The ratio separated cases from controls with an AUC of 0.88 (95% CI 0.82, 0.93). **Conclusion:** A disturbed seminal ferroptosis balance, marked by lower GPX4 and higher ACSL4, tracked with greater sperm DNA damage in asthenozoospermic men. The ACSL4/GPX4 ratio deserves further evaluation as a simple, non-invasive add-on to routine semen work-up.

Keywords: Ferroptosis; Glutathione peroxidase 4; ACSL4; Seminal plasma; Sperm DNA fragmentation; Asthenozoospermia.

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1. INTRODUCTION

Infertility is common, and a male factor plays a part in about 30–50% of couples who cannot conceive [1]. Worldwide, the number of infertile men reached some 56.5 million in 2019, up 76.9% since 1990 [2]. Poor sperm motility, or asthenozoospermia, is one of the main problems found in these men [3]. Its causes run from flagellar gene defects to oxidative stress, and on the oxidative side the mitochondria seem to matter most [4,5].

Ferroptosis is an iron-dependent mortality due to unregulated peroxidation of membrane phospholipids [6–8]. Fundamentally, ferroptosis represents a lack of equilibrium between oxidants and antioxidants [9]. Its threshold is mostly set by two enzymes. GPX4 is a

selenoprotein that reduces phospholipid hydroperoxides to non-toxic alcohols in the presence of glutathione [10,11]. ACSL4 does the opposite. It stimulates arachidonic and adrenic acids, so they can be incorporated into phosphatidylethanolamine, the primary target of peroxidation [12,13]. Its growth is viewed as an indication of ferroptosis sensitivity [14]. Spermatozoa are an easy target. Their membranes are full of polyunsaturated fatty acids, they have little cytoplasm, and GPX4 is a structural protein of the midpiece [15,16]. Sperm from asthenozoospermic men showed low GPX4 with high iron and malondialdehyde [17]. Sperm ACSL4 rose in varicocele and genital infection [18], and varicocele was tied to low GPX4 and a doubled DNA fragmentation index [19].

DNA fragmentation harms both natural and assisted conception [20]. Seminal plasma, with its extracellular vesicles, is a practical source of biomarkers [21], and it can be sampled even when motile sperm are scarce. Still, no study has set seminal-plasma GPX4 and ACSL4 against sperm DNA fragmentation, and none has done so in Iraq, where seminal oxidative stress in infertile men is already documented [22]. We therefore compared seminal GPX4, ACSL4 and their ratio between asthenozoospermic and normozoospermic Iraqi men and tested their link with DNA fragmentation.

2. MATERIALS AND METHODS

2.1. Study design, setting and ethics

This was a case–control study. Men were enrolled at the Fertility Centre, Al-Diwaniyah, Iraq, between January 2025 and September 2025. The protocol was approved by the Research Ethics Committee of the College of Medicine, University of Al-Qadisiyah (approval no. QU-MED-2024-0397, dated 15 November 2024). Every participant gave written informed consent, and the work followed the Declaration of Helsinki. Figure 01 summarises the workflow.

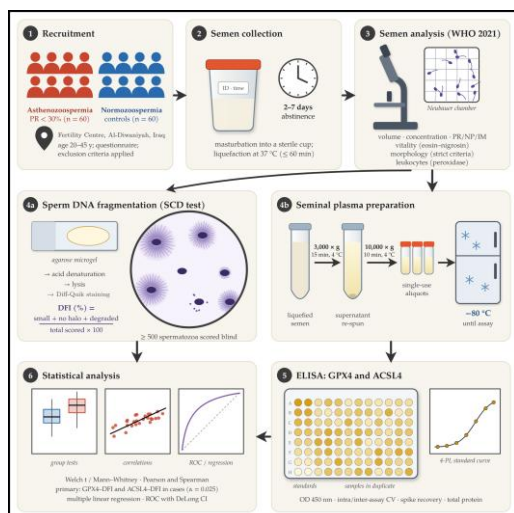


Figure 01: Study workflow. Men were recruited and examined by the WHO 2021 manual (1–3). Each sample was split: one aliquot went to the SCD test for DNA fragmentation (4a), and the rest was centrifuged twice to obtain cell-free seminal plasma, which was stored at -80°C (4b). GPX4 and ACSL4 were measured by sandwich ELISA with total protein (5), and all data were analysed with the pre-set statistical plan (6).

2.2. Participants

Men aged 20–45 years who came for semen analysis were screened. Cases had isolated asthenozoospermia, meaning progressive motility (PR) below 30% while every other semen variable reached the WHO 2021 lower reference limits [23,24]. These limits are 1.4 mL for volume, $16 \times 10^6/\text{mL}$ for concentration, 39×10^6

for total count, 54% for vitality and 4% for normal forms. Total motility was not used as a separate inclusion criterion because it is the arithmetic sum of progressive and non-progressive fractions; when PR falls below 30%, total motility is expected to decrease as well, even when all other parameters are normal. Controls were normozoospermic men seen in the same laboratory over the same period partners of women with a female-factor diagnosis or volunteers who came for premarital screening.

Varicocele and genital infection were ruled out with care, because both raise ferroptosis markers in semen on their own [18,19]. We excluded men with azoospermia or oligozoospermia, leukocytospermia ($> 1 \times 10^6$ peroxidase-positive cells/mL), clinical varicocele, genital tract infection, cryptorchidism, or past testicular surgery or trauma. Men with diabetes, thyroid, liver or kidney disease, or fever in the last 3 months were also left out. So were current smokers, since smoking alone raises seminal iron and lipid peroxidation [25], and men who drank alcohol, worked with heat or chemicals, or had taken antioxidants, hormones or other drugs that affect spermatogenesis in the last 3 months. Age, body mass index (BMI) and abstinence time were recorded on a short questionnaire.

2.3. Semen collection and analysis

Samples were produced by masturbation into sterile wide-mouthed containers after 2–7 days of abstinence, in a room next to the laboratory. They were left to liquefy at 37°C and handled within 60 min. Analysis followed the WHO 2021 manual [23]. Volume was taken by weight, and concentration was counted in an improved Neubauer haemocytometer. Motility was graded as progressive, non-progressive or immotile on 200 spermatozoa in each of two replicates, on a heated stage at 37°C under $\times 400$ phase contrast. Vitality was evaluated using eosin–nigrosin. Morphology was evaluated on Papanicolaou-stained smears using rigorous standards. Leukocytes were counted with the peroxidase test. All readings were performed by two qualified technicians blind to the group in a private room adjacent to the laboratory. If the two replicate counts were not within the permitted limit defined in the handbook, the slide was re-prepared and re-read. The WHO centiles were not employed as rigorous diagnostic cut-offs but as reference distributions, as recommended in the sixth edition [26].

2.4. Preparation of seminal plasma

An aliquot of liquefied semen was first set aside for the DNA fragmentation test. The rest was centrifuged at $3,000 \times g$ for 15 min at 4°C . The supernatant was spun again at $10,000 \times g$ for 10 min to clear remaining cells and debris. The clear seminal plasma was split into single-use aliquots and frozen at -80°C within 30 min of collection. No aliquot was thawed twice, and all samples were assayed within 3 months of collection. The two-step protocol was chosen because the

analytes of interest, GPX4 and ACSL4, are normally intracellular. A single low-speed spin (e.g. $300 \times g$) suffices when the target is a classically secreted protein such as kisspeptin, but it leaves residual cells and large vesicles that can release intracellular contents during freeze-thaw. The added $10,000 \times g$ step removes these, so that the signal measured by ELISA reflects material already released into the fluid rather than artefactual lysis during sample handling.

2.5. Measurement of GPX4, ACSL4 and total protein

GPX4 and ACSL4 were measured with commercial sandwich ELISA kits for human GPX4 (Elabscience, Wuhan, China; catalogue no. E-EL-H1491) and human ACSL4 (Elabscience, Wuhan, China; catalogue no. E-EL-H5765). ELISA has already been used for both proteins in human semen samples [18]. These kits are made for serum and cell lysates, not seminal plasma, so we checked them first. Pooled seminal plasma was spiked with standard to test recovery, and serial dilutions were run to test linearity; the working dilution was 1:5. Samples, standards and blanks were run in duplicate, and absorbance was read at 450 nm on a BioTek ELx800, Agilent Technologies, USA. Concentrations were read from a four-parameter logistic curve. Intra- and inter-assay coefficients of variation were 5.8% and 7.2%, and mean spike recovery was 94%. Values below the lower limit of quantification were set at half that limit (8 samples).

Total protein was measured by the Bradford method, and both markers were also expressed per mg of protein. The ACSL4/GPX4 ratio was calculated for each man as a simple index of ferroptotic pressure. No single protein proves that ferroptosis took place [27], so we treat these values as markers of the pathway and nothing more.

2.6. Sperm DNA fragmentation

DNA fragmentation was measured with the sperm chromatin dispersion (SCD) test (Halosperm G2, Halotech DNA, Madrid, Spain) [20,28]. In brief, liquefied semen was diluted to $10 \times 10^6/\text{mL}$, mixed with low-melting agarose and set on pre-coated slides. The slides were treated with acid denaturant and then with lysis solution, dehydrated in graded ethanol and stained with Diff-Quik stain. At least 500 spermatozoa per sample were scored under bright-field at $\times 400$ – 1000 by one observer who did not know the group. Heads with a large or medium halo were counted as intact. Heads with a small halo, no halo, or no halo and a degraded core were counted as fragmented. The DNA fragmentation index (DFI) is the percentage of fragmented cells. The SCD test is widely used in clinics and has been set against standard semen variables in large series [29,30].

2.7. Sample size

The primary question was how GPX4 and ACSL4 each relate to DFI within the case group. We took $r = 0.40$ as the smallest correlation of practical interest. Because there were two primary tests, α was set at 0.025 (Bonferroni), with 80% power. By the Fisher z method:

$$n = [(z_{1-\alpha/2} + z_{1-\beta}) / C]^2 + 3, \text{ where } C = 0.5 \ln[(1 + r)/(1 - r)]$$

With $z_{0.9875} = 2.241$, $z_{0.80} = 0.842$ and $C = 0.424$, Eq. (1) gives $n = 56$. We enrolled 60 cases to allow for unusable samples, which gives 83% power for $r = 0.40$. With 60 men in each group, the study also has 80% power to detect a standardised group difference of 0.52 at $\alpha = 0.05$.

2.8. Statistical analysis

Normality was checked with the Shapiro–Wilk test and Q–Q plots. Data are given as mean $\pm$ SD, with median and interquartile range for skewed variables. Groups were compared with Welch's t -test, which does not assume equal variances; the mean difference is shown with its 95% confidence interval (CI) and with Hedges' g . The Mann–Whitney U test was run as a check. Correlations were tested with Pearson's r , with 95% CIs from Fisher's z , and repeated with Spearman's ρ . The two primary correlations were judged at $\alpha = 0.025$. All other tests were exploratory, used $\alpha = 0.05$ and were not corrected. The analysis used complete cases; no values were missing. The eight readings below the lower limit of quantification were replaced by half that limit, as described in Section 2.5. Outlying values were checked against the laboratory records and were removed only when a technical error was documented.

Multiple linear regression modelled DFI in cases, with GPX4, ACSL4, age, BMI and abstinence as predictors; collinearity was checked with the variance inflation factor (VIF). Receiver operating characteristic (ROC) curves were drawn for GPX4, ACSL4, their ratio and a logistic model holding both markers. The area under the curve (AUC) is given with a DeLong 95% CI, and cut-offs were set by the Youden index. Analyses were verified in IBM SPSS Statistics version 28 (IBM Corp., Armonk, NY, USA) and MedCalc version 22 (MedCalc Software, Ostend, Belgium).

3. RESULTS AND DISCUSSION

3.1. Characteristics of the participants

Table 1 lists the demographic and semen data. The groups were similar in age, BMI and abstinence time (all $p > 0.05$). This point matters, because each of these can shift DFI on its own [29]. As the case definition requires, progressive and total motility were much lower in the asthenozoospermic men. Vitality was 54.2% against 71.5% ($p < 0.001$), and sperm concentration was 58.7 against $64.2 \times 10^6/\text{mL}$ ($p = 0.237$). Concentration, volume, total count and vitality all stayed above the WHO lower limits in both groups

by design [23,24]. Total motility in the case group averaged 39.9%, below the 42% fifth-centile reference, but this is an expected arithmetic consequence of the defining PR deficit rather than an independent abnormality. Normal morphology was significantly lower in cases (5.1% vs 6.4%; $p = 0.010$), yet both group means exceeded the 4% WHO threshold, so the statistical difference reflects a distributional shift within the normal range rather than frank teratozoospermia. We chose isolated asthenozoospermia on purpose. Men with low counts or poor morphology often carry several defects at once, and mixing them would make any marker hard to read.

Table 01: Demographic and semen characteristics of the study groups.

Variable	Controls (n = 60)	Asthenozoospermia (n = 60)	Mean difference (95% CI)	p value
Age (years)	31.4 ± 5.2	32.1 ± 5.6	0.7 (-1.26, 2.66)	0.478
BMI (kg/m ²)	26.3 ± 3.1	26.8 ± 3.4	0.5 (-0.68, 1.68)	0.402
Abstinence (days)	3.6 ± 1.1	3.8 ± 1.3	0.2 (-0.24, 0.64)	0.365
Semen volume (mL)	3.1 ± 1.3	2.8 ± 1.2	-0.3 (-0.75, 0.15)	0.192
Sperm concentration (× 10 ⁶ /mL)	64.2 ± 26.5	58.7 ± 23.8	-5.5 (-14.65, 3.65)	0.237
Total sperm number (× 10 ⁶ /ejaculate)	199.0 ± 108.5	164.4 ± 95.2	-34.6 (-71.71, 2.51)	0.068
Progressive motility (%)	51.8 ± 9.4	17.6 ± 6.8	-34.2 (-37.13, -31.27)	<0.001
Non-progressive motility (%)	14.5 ± 5.8	22.3 ± 7.4	7.8 (5.38, 10.22)	<0.001
Total motility (%)	66.3 ± 9.2	39.9 ± 9.5	-26.4 (-29.78, -23.02)	<0.001
Immotile spermatozoa (%)	33.7 ± 9.2	60.1 ± 9.5	26.4 (23.02, 29.78)	<0.001
Vitality (%)	71.5 ± 8.3	54.2 ± 11.6	-17.3 (-20.93, -13.67)	<0.001
Normal forms (%)	6.4 ± 2.6	5.1 ± 2.8	-1.3 (-2.28, -0.32)	0.010

Values are mean ± SD. Difference = asthenozoospermia – controls. p from Welch's t-test; the Mann–Whitney U test gave the same conclusions.

3.2. Seminal-plasma GPX4, ACSL4 and the ACSL4/GPX4 ratio

Marker levels are shown in Table 2 and Figure 2a–c. Seminal-plasma GPX4 was significantly lower in the asthenozoospermic group (5.18 ± 1.76 vs 8.52 ± 2.34 ng/mL; difference -3.34, 95% CI -4.09, -2.59; $p < 0.001$; $g = -1.60$). ACSL4 was significantly higher (6.37 ± 1.88 vs 3.84 ± 1.25 ng/mL; $p < 0.001$; $g = 1.57$). The ratio, which puts both signals together, was 1.36 ± 0.51 against 0.48 ± 0.20 ($p < 0.001$). Expressing the markers per mg of protein did not change these results.

Table 02: Seminal-plasma ferroptosis markers and sperm DNA fragmentation in the two groups.

Marker	Controls (n = 60)	Asthenozoospermia (n = 60)	Difference (95% CI)	Hedges' g	p value
GPX4 (ng/mL)	8.52 ± 2.34	5.18 ± 1.76	-3.34 (-4.09, -2.59)	-1.60	<0.001
GPX4 (ng/mg protein)	0.421 ± 0.118	0.258 ± 0.092	-0.163 (-0.201, -0.125)	-1.53	<0.001
ACSL4 (ng/mL)	3.84 ± 1.25	6.37 ± 1.88	2.53 (1.95, 3.11)	1.57	<0.001
ACSL4 (ng/mg protein)	0.190 ± 0.063	0.318 ± 0.098	0.128 (0.098, 0.158)	1.54	<0.001
ACSL4/GPX4 ratio	0.48 ± 0.20	1.36 ± 0.51	0.88 (0.74, 1.02)	2.25	<0.001
DFI (%)	15.3 ± 5.8	32.7 ± 10.4	17.4 (14.35, 20.45)	2.05	<0.001

Values are mean ± SD. p from Welch's t-test. GPX4 and ACSL4 are expressed in ng/mL; protein-normalised values in ng/mg protein.

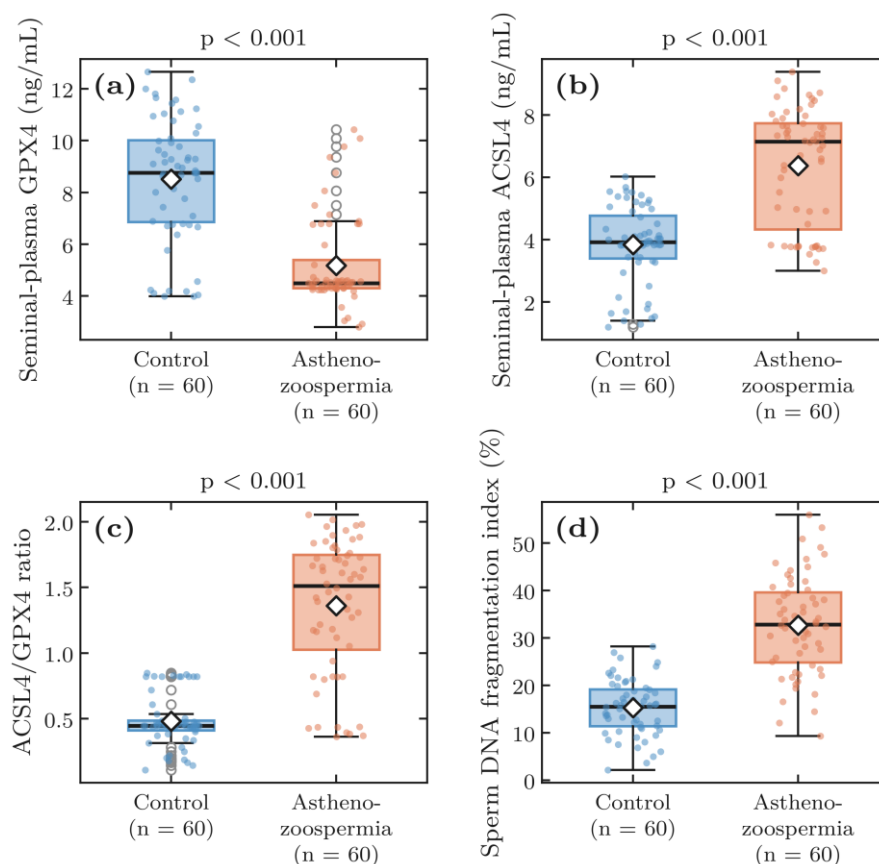


Figure 02: Seminal-plasma GPX4 (a), ACSL4 (b), the ACSL4/GPX4 ratio (c) and the sperm DNA fragmentation index (d) in controls and asthenozoospermic men. Boxes show the median and interquartile range, whiskers extend to $1.5 \times$ IQR, diamonds mark the mean, and points are individual men; p values are from Welch's t-test.

How should a seminal-plasma value be read? GPX4 and ACSL4 are intracellular enzymes, not classic secreted proteins. Their presence in cell-free seminal plasma therefore requires explanation. Three non-exclusive sources are plausible: (i) release from damaged or apoptotic spermatozoa whose membranes have lost integrity, (ii) active secretion via extracellular vesicles (prostatosomes, epididymosomes) that carry cytoplasmic cargo including GPX4 [21,31], and (iii) low-level lysis of residual germ cells or leukocytes during ejaculation and liquefaction. Our two-step centrifugation ($3,000$ then $10,000 \times g$) was designed to remove intact cells and large vesicles, so the ELISA signal comes mainly from truly soluble or small-vesicle-associated protein rather than from cells that broke open during handling. We did not measure a cytoplasmic lysis marker such as lactate dehydrogenase (LDH); future studies should include LDH to quantify any residual lysis contribution and, ideally, fractionate seminal plasma by ultracentrifugation to separate soluble from vesicle-bound GPX4 and ACSL4. A seminal level is therefore a composite signal that reflects what the reproductive tract releases and what the sperm lose. That is also its practical appeal, since it can be measured without isolating sperm, even when few motile cells are present. Earlier seminal-plasma work on this pathway is limited. Seminal plasma of heavy smokers held more iron and lipid ROS and less glutathione [25], and seminal ferritin and F_2 -isoprostanes were raised in varicocele and infection [18].

These statistics are set according to the literature on sperm-cells. According to Hao et al. [17], GPX4 and SLC7A11 were significantly reduced in sperm from 42 asthenozoospermic men with increased iron, ROS and malondialdehyde. In an Italian series, sperm GPX4 was not different between fertile and infertile men, but ACSL4 increased in both varicocele and infection and paralleled seminal F_2 -isoprostanes [18]. The authors named the image ACSL4-driven and GPX4-independent. Both markers were transported in our seminal plasma, GPX4 decreased and ACSL4 increased, suggesting a mixed pattern rather than a strictly ACSL4 driven one. The elevation in ACSL4 is compatible with the idea that upregulation of ACSL4 indicates cells ready for ferroptosis [12, 14]. A reduction in GPX4, the selenoenzyme which makes up almost half of the protein in the midpiece capsule [11, 15], would result in its loss. Laboratory work is pointing the same way. Arachidonic acid oxidative stress reduced GPX4, increased ACSL4 and reduced human sperm velocity [32]. GPX4 was reduced in human testis with increasing severity of spermatogenic damage [33].

Why bother with a ratio? Ferroptosis requires the availability of peroxidisable phospholipids, iron to initiate the chain reaction and the failure of repair [10]. ACSL4 addresses the first of these needs, and GPX4 the last [13]. A man with high ACSL4 but plenty of GPX4 may be well protected, and vice versa. The ratio attempts to express that imbalance as

a single figure. It has a practical side as well. The ratio cancels out much of the difference in dilution between ejaculates with or without total protein, since both proteins are from the same fluid.

3.3. Sperm DNA fragmentation

The DFI was $32.7 \pm 10.4\%$ in men with asthenozoospermia and $15.3 \pm 5.8\%$ in controls (difference 17.4%, 95% CI 14.35 to 20.45; $p < 0.001$) (Figure 2d). 38 (63.3%) patients had DFI > laboratory standard of 30%. The association between low motility and DNA damage is widely established. In a series of 2,567 samples examined by the same SCD method, DFI was inversely correlated with progressive motility ($r = -0.257$) [29], and an inverse relationship was also noted in another large series [30]. In the varicocele trial, DFI was 22.07% against 10.78% in controls, with low GPX4 and high seminal iron [19]. The DFI cut-offs are assay and laboratory dependent and hence we considered DFI as a continuous variable rather than dichotomise into high and low DFI groups [28].

The mechanism is in agreement with the ferroptosis concept. Hydrogen peroxide generated by the midpiece can reach the nucleus where oxidised bases are removed. This leads to abasic sites and, with increasing stress, strand breaks [34]. Lipid peroxidation provides a second hit. Its aldehydes, 4-hydroxynonenal and malondialdehyde, interact to DNA and proteins to create adducts [16, 35]. The spermatozoon has very little cytoplasm and minimal healing capacity. So the injury is likely to remain.

3.4. GPX4, ACSL4 and DFI in the case group (primary analysis)

Table 3 and Figure 3 show the primary analysis. Among asthenozoospermic men, DFI correlated with GPX4 at $r = -0.44$ (95% CI $-0.62, -0.21$; Bonferroni $p = 0.0008$) and with ACSL4 at $r = 0.41$ (95% CI $0.17, 0.60$; Bonferroni $p = 0.002$). The ratio correlated with DFI at $r = 0.52$ ($p < 0.001$). GPX4 and ACSL4 were inversely related to each other ($r = -0.38$). Spearman coefficients were close to the Pearson values throughout.

Table 03: Correlations of seminal-plasma GPX4, ACSL4 and their ratio with sperm DNA fragmentation.

Pair	Group	n	Pearson r (95% CI)	p value	Bonferroni p	Spearman ρ
GPX4 vs DFI (primary)	Cases	60	$-0.44 (-0.62, -0.21)$	0.0004	0.0008	-0.41
ACSL4 vs DFI (primary)	Cases	60	$0.41 (0.17, 0.60)$	0.001	0.002	0.38
ACSL4/GPX4 vs DFI	Cases	60	$0.52 (0.31, 0.68)$	<0.001	—	0.49
GPX4 vs ACSL4	Cases	60	$-0.38 (-0.58, -0.14)$	0.003	—	-0.35
GPX4 vs DFI	Controls	60	$-0.16 (-0.40, 0.10)$	0.222	—	-0.14
ACSL4 vs DFI	Controls	60	$0.13 (-0.13, 0.37)$	0.322	—	0.11
GPX4 vs DFI	Pooled	120	$-0.61 (-0.71, -0.49)$	<0.001	—	-0.57
ACSL4 vs DFI	Pooled	120	$0.63 (0.51, 0.73)$	<0.001	—	0.54

95% CIs from Fisher's z. Bonferroni correction applies to the two primary tests only; the others are exploratory. Pooled estimates mix the group difference into the association and are shown for comparison.

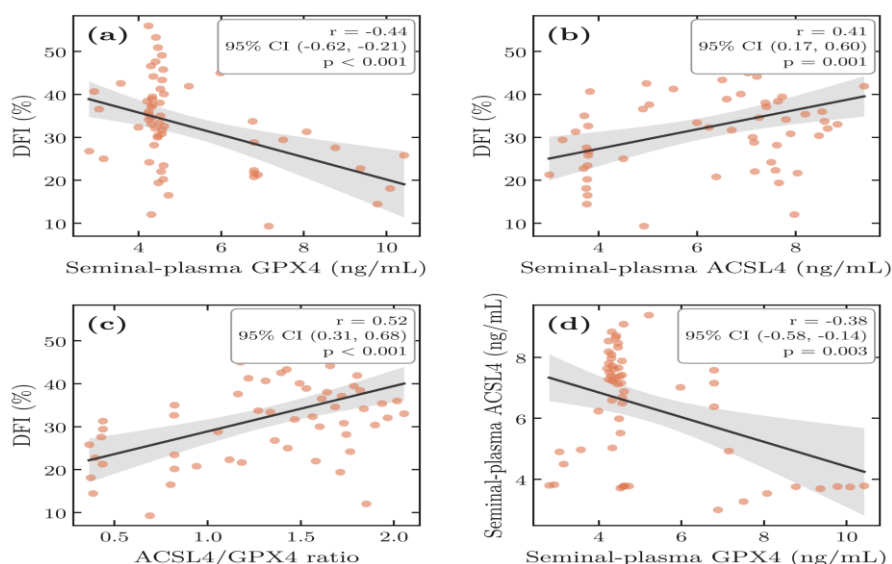


Figure 03: Scatter plots in the asthenozoospermic group ($n = 60$): DFI against seminal-plasma GPX4 (a), ACSL4 (b) and the ACSL4/GPX4 ratio (c), and ACSL4 against GPX4 (d). Lines are least-squares fits with 95% confidence bands; r and p are Pearson values.

Correlations inside one group are usually weaker than pooled ones. Pooling cases and controls folds the group difference into the association, which is why the pooled values in Table 3 are shown only for comparison. The inverse link between GPX4 and DFI fits the protective role of this selenoenzyme: men whose seminal plasma carried less GPX4 tended to have more fragmented sperm DNA. The positive link between ACSL4 and DFI is consistent with a greater supply of peroxidisable substrates fuelling lipid-mediated DNA damage. A mechanism for the GPX4 link is available. The nuclear form of GPX4 supports chromatin condensation, and raising it cut phthalate-induced DNA damage in male germ cells [36]. The only clinical study we found that reported ferroptosis markers and DFI in the same men showed low GPX4 together with high DFI in varicocele [19]. Even so, a modest correlation in a cross-sectional design cannot show that ferroptosis breaks sperm DNA. Faulty chromatin packing and abortive apoptosis also raise DFI [20]. Both correlations are moderate rather than strong, which is what this design leads one to expect. All cases were selected for low motility, and this narrows the spread of several variables inside the group. A narrow range weakens any correlation, whatever the biology. For the same reason the control and pooled estimates in Table 3 should not be read as tests of the hypothesis. They only show how the association behaves across the full range of semen quality.

3.5. Relation to conventional semen variables

Figure 4 shows the Spearman matrix in cases. GPX4 correlated with progressive motility at $\rho = 0.36$ and with vitality at $\rho = 0.33$, and ACSL4 correlated with progressive motility at $\rho = -0.31$. The ACSL4/GPX4 ratio correlated negatively with progressive motility ($\rho = -0.35$) and with vitality ($\rho = -0.14$), strengthening its value as a composite index of ferroptotic pressure. Hao et al. reported a similar positive link between sperm GPX4 and progressive motility ($r = 0.397$) [17], while ACSL4 moved against semen quality in the Italian series [18]. Motility depends on an intact mitochondrial sheath and a steady ATP supply. Oxidative damage to either will slow the cell well before it kills it [5]. Vitality, as read by eosin exclusion, is a test of membrane integrity. It is therefore the semen variable closest to the final step of ferroptosis, in which peroxidised membranes lose their barrier function and rupture [8].

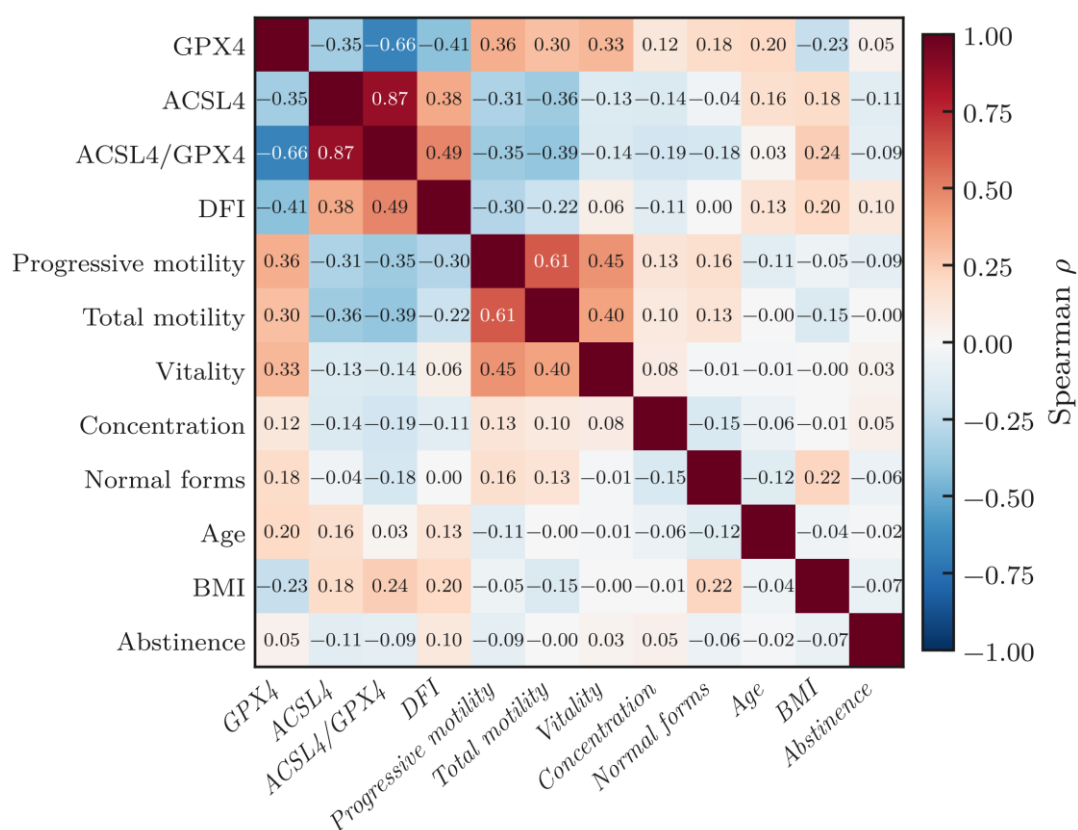


Figure 04: Spearman correlation matrix of seminal ferroptosis markers, DFI, semen variables and covariates in the asthenozoospermic group.

3.6. Multivariable model

In the regression model for DFI in cases (Table 4), GPX4 had a standardised β of -0.31 ($p = 0.020$) and ACSL4 a β of 0.26 ($p = 0.045$) after adjustment for age, BMI and abstinence. The model explained 29% of the variance in DFI (adjusted $R^2 = 0.23$). All VIF values were below 2.0, so the two markers did not duplicate each other. GPX4 retained a significant independent contribution to DFI after adjustment, while ACSL4 added modestly, confirming that the two markers capture partly separate aspects of the ferroptosis axis. Age and abstinence were kept in the model on prior

grounds, not because they reached significance. Both show small but consistent links with DFI in large clinical series [29], and leaving them out could bias the marker coefficients.

Table 04: Multiple linear regression of DFI in asthenozoospermic men (n = 60).

Predictor	B (95% CI)	Standardised β	p value	VIF
GPX4 (ng/mL)	-1.82 (-3.35, -0.29)	-0.31	0.020	1.28
ACSL4 (ng/mL)	1.45 (0.04, 2.86)	0.26	0.045	1.24
Age (years)	0.22 (-0.22, 0.66)	0.12	0.317	1.05
BMI (kg/m ²)	0.35 (-0.38, 1.08)	0.11	0.341	1.08
Abstinence (days)	0.68 (-1.19, 2.55)	0.08	0.468	1.03
Model	$R^2 = 0.29$	Adjusted $R^2 = 0.23$	F-test p = 0.002	–

B, unstandardised coefficient; VIF, variance inflation factor.

3.7. Discrimination between cases and controls

ROC results are given in Table 5 and Figure 5. The AUC was 0.82 for GPX4, 0.80 for ACSL4, 0.88 for the ratio and 0.91 for the two-marker model. At the Youden cut-off of 0.82, the ratio had a sensitivity of 83.3% and a specificity of 81.7%. These values come from the same men used to set the cut-offs, so they are optimistic. They need testing in a separate group before any clinical use. It is also fair to say what the ROC analysis is not for. Asthenozoospermia is diagnosed by looking at motile sperm, and no seminal protein will replace that. The AUC is used here as a scale-free measure of how far apart the two groups sit. If the markers have a clinical role, it is more likely to be in sorting men with poor motility by the mechanism behind it.

Table 05: ROC analysis of seminal-plasma markers for separating asthenozoospermic men from controls.

Marker	AUC (95% CI)	Cut-off	Sensitivity (%)	Specificity (%)	Youden J
GPX4 (case if $\leq$ cut-off)	0.82 (0.75, 0.89)	≤ 6.75 ng/mL	76.7	78.3	0.55
ACSL4 (case if $\geq$ cut-off)	0.80 (0.72, 0.87)	≥ 4.90 ng/mL	73.3	75.0	0.48
ACSL4/GPX4 ratio	0.88 (0.82, 0.93)	≥ 0.82	83.3	81.7	0.65
GPX4 + ACSL4 (logistic model)	0.91 (0.86, 0.95)	≥ 0.58	85.0	86.7	0.72

AUC 95% CI by the DeLong method; cut-offs by the Youden index. For the logistic model the cut-off is a predicted probability.

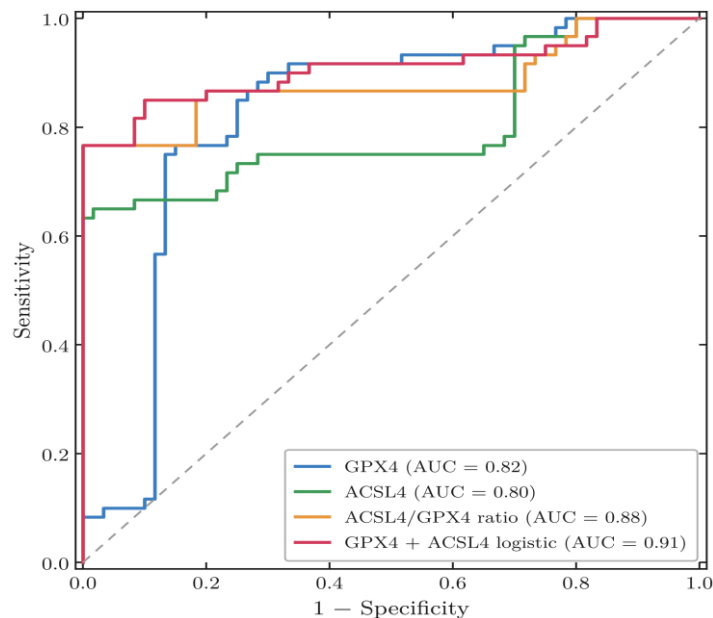


Figure 05: ROC curves for GPX4 (lower values = case), ACSL4, the ACSL4/GPX4 ratio and a logistic model with both markers; the dashed line marks chance.

3.8. Comparison with earlier studies

Table 6 sets our design beside earlier human work. Most studies measured markers inside sperm, not in seminal plasma, and only one measured DFI [19]. The groups differ as well. Varicocele, infection, smoking and azoospermia have been studied more often than isolated asthenozoospermia [18,19,25,33]. Animal models add a causal layer that human studies lack. Busulfan, zearalenone, arsenite, bisphenol A, fine particulate matter and diabetes each set off ferroptosis in

the testis, with iron build-up and lipid peroxidation, and harmed sperm output or motility; most of them also lowered GPX4 or SLC7A11 [37–42]. In several of these models, ferrostatin-1 or iron chelation reversed the damage [37,38,42]. The evidence is not uniform, though. In a rat varicocele model, iron built up in the testis while GPX4 and the other ferroptosis markers did not change [43]. Recent reviews treat ferroptosis as an emerging, not yet settled, player in male infertility [44,45], and our seminal data should be read against this mixed picture.

Table 06: Human studies of ferroptosis markers in male infertility (2020–2026) compared with the present study.

Study (country)	Groups (n)	Sample	Markers and method	Main findings
Ou et al., 2020 [25] (China)	Heavy smokers 70; non-smokers 75	Seminal plasma; GC-2spd cells	Iron, lipid ROS, GSH; GPX4 (western blot, cells)	Smokers: lower GSH, higher lipid ROS and iron, poorer vitality and PR; smoke extract lowered GPX4 in cells, reversed by ferrostatin-1
Hao et al., 2023 [17] (China)	Asthenozoospermia 42; normozoospermia 45	Sperm	ROS, MDA, iron, MMP; GPX4 and SLC7A11 expression	ROS, MDA and iron higher; MMP, GPX4 and SLC7A11 lower; GPX4 vs PR $r = 0.397$
Sun et al., 2023 [19] (China)	Controls 20; bilateral varicocele 26	Seminal plasma and sperm	ROS, iron, GSH, T-SOD, CAT; GPX4 mRNA/protein; DFI	ROS and iron higher; GSH, SOD, CAT and GPX4 mRNA lower; DFI 22.07% vs 10.78%
Moretti et al., 2024 [18] (Italy)	Fertile 19; infection 15; varicocele 13	Seminal plasma; sperm lysate	Ferritin, transferrin, F ₂ -IsoPs; GPX4 and ACSL4 by ELISA	ACSL4 higher in both infertile groups; GPX4 unchanged; F ₂ -IsoPs and ferritin higher; DFI not measured
Contreras-Mellado et al., 2025 [32] (Chile)	Normozoospermic donors (in vitro)	Sperm	Arachidonic acid stress; flow cytometry and confocal microscopy	GPX4 lower; ACSL4, SLC7A11, IREB2, Fe ²⁺ and lipid peroxidation higher; motility and viability lower
Romac et al., 2026 [33] (Croatia)	Obstructive 14; non-obstructive azoospermia 31	Testis (TESE)	ALOX15 and GPX4 by immunofluorescence	Both higher in obstructive azoospermia; GPX4 lowest in severe hypospermatogenesis
Present study (Iraq)	Asthenozoospermia 60; normozoospermia 60	Seminal plasma; sperm (SCD)	GPX4 and ACSL4 by ELISA; ACSL4/GPX4 ratio; DFI	GPX4 lower and ACSL4 higher in asthenozoospermia; ACSL4/GPX4 ratio elevated; GPX4 inversely and ACSL4 positively correlated with DFI; ratio showed good diagnostic accuracy

MMP, mitochondrial membrane potential; MDA, malondialdehyde; F₂-IsoPs, F₂-isoprostanes; PR, progressive motility; TESE, testicular sperm extraction.

3.9. Clinical meaning and the Iraqi setting

DNA fragmentation testing is advised in several clinical settings, but how to treat a high DFI is still debated [28,46]. In a global survey, 76.9% of experts gave empiric antioxidants for raised DFI [46]. A seminal marker of ferroptotic pressure might help to pick the men most likely to benefit, though only trials can show this. Iraqi studies have already found low seminal antioxidant capacity in infertile men [22]. In two Iraqi cohorts, coenzyme Q10 raised seminal total antioxidant capacity and lowered both ROS and DFI [47,48]. Reduced CoQ10 is also the radical-trapping antioxidant of the FSPI pathway, which guards membranes independently of GPX4 [49]. That overlap deserves a direct test. Future Iraqi studies should also record environmental and occupational exposures in more detail. Several of the agents that trigger testicular ferroptosis in animals, such as arsenite, bisphenol A

and fine particulate matter, are common pollutants [39–41]. Ferroptosis also crosses paths with other death programmes in the testis, such as pyroptosis and autophagy-linked death, which may blur the reading of any single marker [50].

3.10. Strengths and limitations

The study has some clear strengths. The groups were matched on the main confounders, the primary hypotheses and α were fixed in advance, and the ELISA kits were checked in seminal plasma before use. There are also limits. The design is cross-sectional and from one centre, so it cannot show cause or direction. Seminal levels do not tell us how much of each enzyme sits inside the sperm, and without a lysis marker such as LDH we cannot distinguish protein released by physiological vesicle secretion from protein released by cell damage. The two-step centrifugation removes

intact cells but not small extracellular vesicles, so the measured concentrations are a composite of soluble and vesicle-associated fractions. We did not measure iron, lipid peroxidation products or GPX4 activity, and current guidance asks for such a panel before ferroptosis is claimed [27]. The SCD test reads DNA breaks after denaturation and may not agree with TUNEL or SCSA in every sample [20]. Genetic causes of poor motility were not screened [4]. Each man gave one semen sample, although semen quality varies from one ejaculate to the next. Control men were normozoospermic but were not proven fertile, and reproductive outcomes were not followed.

4. CONCLUSION

This study looked at two ferroptosis enzymes in the seminal plasma of asthenozoospermic and normozoospermic Iraqi men and set them against sperm DNA fragmentation. Asthenozoospermic men showed significantly lower GPX4, significantly higher ACSL4 and a markedly higher ACSL4/GPX4 ratio than controls, and their sperm carried significantly more DNA fragmentation. Within the case group, DFI was inversely related to GPX4 and positively related to ACSL4. These findings support the idea that a shift in the seminal ferroptosis balance goes with DNA damage in poorly motile sperm. Seminal plasma is easy to collect and store, so the ratio could become a simple add-on to routine semen work-up. It is not ready for clinical use yet. Larger, multicentre studies should measure both enzymes inside sperm as well as in seminal plasma, include a lysis marker such as LDH to confirm that the seminal-plasma signal is not driven by cell damage during handling, add iron and lipid peroxidation markers, and follow couples to pregnancy. Trials of antioxidant or ferroptosis-targeted treatment in men with high DFI would then show whether this pathway can be changed.

5. AUTHOR CONTRIBUTIONS

Durar Kamil Jabbar: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration.

6. FUNDING

This research received no external funding.

7. CONFLICTS OF INTEREST

The author declares no conflict of interest.

8. DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author on reasonable request. The analysis script is provided as supplementary material.

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