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Human papillomavirus and male infertility correlation analysis following World Health Organization 2021 guidelines

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Male infertility is a complex issue influenced by multiple environmental and pathological factors. In this context, the impact of Human papillomavirus (HPV) infection on male fertility remains controversial. The introduction of new WHO 2021 evaluation criteria, included in the 6th ed. of Laboratory Manual for the examination and processing of human semen, i.e. DNA fragmentation index (DFI), slow and rapid progressive motility, could provide additional information about this correlation. 121 semen samples of male partners of HPV-positive women attending In Vitro Fertilization (IVF) were evaluated following WHO 2021 and HPV-DNA test. Comparing HPV-negative and positive samples for rapid and slow progressive motility showed significantly different results ($p = 0.0018$, $p = 0.0004$), contrary to what was observed for total progressive motility. Regarding sperm DFI, only high-risk HPV infections affected DNA integrity. In addition, the correlation between the different semen parameters revealed a significant correlation between midpiece morphological defects and rapid progressive motility in the HPV-positive group (rho = 0.43, $p = 0.0006$). In conclusion, WHO 2021 provides additional information regarding HPV's impact on seminal parameters. The correlation between HPV positivity, midpiece defects and a higher rapid progressive motility opens new research perspectives that may help unravel the issues surrounding the role of HPV in compromising sperm quality.

Keywords Male infertility, HPV, WHO 2021, DFI, Rapid progressive motility, Slow progressive motility

A thorough and up-to-date assessment of semen quality is crucial in understanding the reasons behind male infertility. In this regard, the World Health Organization (WHO) has been working since 1980 to develop appropriate guidelines for the examination and processing of human semen[1], with the 6th and latest edition[2] having revised methods and criteria from the 2010 version[3,4].

Changes introduced in the latest manual include new minimum values for sperm concentration, viability and morphology, DNA integrity as a new parameter, and the reintroduction of the classification of sperm motility into fast progressive motility, slow progressive motility, non-progressive motility, and immotile sperms[1,4].

All these updates could potentially help untangle controversial topics, such as the influence of Human Papillomavirus (HPV) infection on male fertility.

The risk of contracting HPV at least once in a lifetime is about 80% for any sexually active person[5–7]. More than 200 different HPV genotypes have been identified, divided into high-risk (hrHPV) and low-risk HPV (lrHPV), depending on the likelihood of inducing the development of neoplastic lesions. The International Agency for Research on Cancer (IARC) has built this distinction based on epidemiological, biological, and biochemical evidence[8]. Beyond the extensive role of HPV in the etiopathogenesis of malignancies, studies focused on male HPV infections have shown the presence of HPV DNA in semen and other genital sites such as the penile shaft, glans, corona, scrotum, testis, and perianal and anal regions[9,10]. Talking especially about the

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single spermatozoon, HPV virions have been localised in the equatorial segment of the sperm head, as well as in the midpiece[11–13].

The correlation between HPV infection and its potential impact on semen quality and male reproductive health, as well as natural and assisted reproduction, has been extensively studied but remains controversial: to date, literature has failed to provide consistent results regarding the alteration of sperm parameters in the presence of HPV[14–16].

For example, while Foresta et al.[17], Moghimi et al.[15], Boeri et al.[18], and Yang et al.[19] described a significant decrease in progressive motility rates in HPV-positive patients, others[20–22] found no significant difference between HPV-positive and -negative patients and seminal parameters, as progressive motility. Although, Capra et al.[22] showed how HPV-positive patients had progressive motility below the reference values identified according to the WHO 2010 classification. Against this background, the reintroduction of motility categorisation provides an important opportunity to resolve this controversy.

Two other essential aspects highlighted in the WHO 2021 edition are the importance of assessing sperm DNA fragmentation (SDF) and the role of oxidative stress in semen[1], which can be triggered by genetic disorders, ageing, lifestyle factors, including smoking or diet, and sexually transmitted infections (STIs)[22–24]. The relevance of using the sperm DNA fragmentation index (DFI) as a possible predictor of male fertility lies in the high percentage of double-strand or single-strand DNA breaks reported in the semen of potentially infertile men, even in the absence of other impairments[25,26]. Since intact sperm DNA affects proper embryo development, implantation, and pregnancy outcomes, detecting damaged DNA in semen is essential for sperm quality appraisal and estimate of male reproductive function[22,25,26]. Currently, there is evidence in the literature reporting an inverse correlation between DFI and standard semen parameters such as concentration, motility, and morphology[25]. In light of this, old and new techniques are recommended to assess sperm DNA integrity, including sperm chromatin structure assay, sperm chromatin dispersion (SCD) test, aniline/toluidine blue staining, acridine orange assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay and Comet assay[1,25]. Even concerning HPV-induced damage of sperm DNA, results are inconclusive. A recent meta-analysis showed evidence of sperm DFI greater than 30% in men with seminal HPV infection[27]. On the other hand, Cortés-Gutiérrez et al.[28] and Kaspersen et al.[29] found no association between a DFI > 30% and the presence of seminal HPV DNA.

As it is essential to gain a clearer insight into the correlation between HPV infection and male infertility, we followed up on the updates of the WHO 2021 manual aiming to investigate parameters such as rapid progressive motility, and slowly progressive motility in comparison to progressive motility, in male partners of HPV-positive women of infertile couples.

Results

The entire cohort consisted of 121 male partners of HPV-positive women undergoing IVF, whose mean age was 38.6 ± 8.1 (SD).

As shown in Table 1, semen samples were evaluated for sperm parameters such as DFI, sperm concentration expressed both in ml and total sperm number, motility parameters such as rapid progressive motility, slow progressive motility, non-progressive, immotile, and derived parameters such as progressive motility (rapid progressive motility and slow progressive motility) and total motility (rapid progressive motility, slow progressive motility and non-progressive). Morphological parameters, i.e., normal forms, head defects, neck and midpiece defects and tail defects were also evaluated.

Of the total samples, 61 (50.4%) were negative for HPV. 60 (49.6%) were positive (Table 1).

The parameters mentioned above were distinguished and compared in the two HPV-negative and HPV-positive groups (Table 1). Significant differences were found for rapid and slow progressive motility (median: 5vs10, $p = 0.0018$, median: 25vs15, $p = 0.0004$, respectively), non-progressive motility (median: 10vs20, $p = 0.0401$), normal form (median: 9vs6, $p = 0.015$), neck and midpiece defects (median: 6vs25, $p < 0.0001$), and tail defects (median: 2vs21, $p < 0.0001$).

In particular, the rates of slow progressive motility and normal forms were significantly higher in the HPV-negative group. Conversely, we found a significantly higher percentage of rapid progressive motility, non-progressive motility, neck and midpiece defects, and tail defects in the HPV-positive group.

In Table 2 we reported the multiple logistic regression between the binary variable HPV (positive/negative) and the significant variables of Table 1 such as rapid progressive motility, slow progressive motility, non-progressive, normal forms, neck and midpiece defects, and tail defects. In this step, the HPV variable was defined as a dichotomous variable (0 = negative, 1 = positive). From multivariate analysis, significant positive predictors of HPV were rapid progressive motility% (OR = 1.09, $p = 0.0148$) and neck and midpiece defects (OR = 1.11, $p = 0.0026$), while the only significant negative predictor was slow progressive motility (OR = 0.94, $p = 0.0168$).

For both the HPV-negative and HPV-positive groups, the Spearman correlation coefficient rho was used to evaluate the degree of association between rapid progressive motility, slow progressive motility, progressive motility and all the other seminal parameters already cited above (Table 3).

In the HPV-negative group, a significant negative correlation was found between DFI and rapid progressive motility (rho=-0.30, $p = 0.0182$), slow progressive motility (rho=-0.54, $p < 0.0001$), and progressive motility (rho=-0.51, $p < 0.0001$), as well as between immotile sperm and rapid progressive motility (rho=-0.53, $p < 0.0001$), slow progressive motility (rho=-0.58, $p < 0.0001$), and progressive motility (rho=-0.66, $p < 0.0001$). In contrast, a significant positive correlation was found between sperm concentration/ml and rapid progressive motility (rho = 0.42, $p = 0.0008$), slow progressive motility (rho = 0.55, $p < 0.0001$), and progressive motility (rho = 0.54, $p < 0.0001$), and between normal forms and rapid progressive motility (rho = 0.27, $p = 0.0359$), slow progressive motility (rho = 0.49, $p = 0.0001$), and progressive motility (rho = 0.44, $p = 0.0003$) (Table 3).

Parameters	Total	HPV-negative	HPV-positive	Negative vs. positive, *p*-value (test)
Patients	121	61	60	
Sperm DFI%	27.6 ± 13.4 26 (17.4–34.3)	27.4 ± 13.2 24.5 (18.6–33)	27.9 ± 13.6 27 (16–38)	$p = 0.71$ (MW)
Sperm concentration/ml	46.5 ± 48.8 29 (12.3–60.5)	50.9 ± 55.0 30 (7.8–70)	42.0 ± 41.6 28 (15–54)	$p = 0.86$ (MW)
Rapid progressive motility %	10.5 ± 0.9 5 (2.8–20)	7.5 ± 7.7 5 (0.0–15)	13.6 ± 11.0 10 (5–20)	$p = 0.0018$* (MW)
Slow progressive motility %	18.4 ± 10.3 20 (10–25)	21.4 ± 11.0 25 (14.5–30)	15.4 ± 8.6 15 (10–20)	$p = 0.0004$* (MW)
Progressive motility %	28.9 ± 15.1 30 (20–40)	28.9 ± 15.6 35 (15–40)	28.9 ± 14.8 30 (20-38.5)	$p = 0.56$ (MW)
Non-progressive %	17.5 ± 9.3 15 (10–25)	16.5 ± 10.9 10 (10–20)	18.6 ± 7.3 20 (14.5–25)	$p = 0.0401$* (MW)
Total motility %	46.4 ± 16.3 50 (40–55)	45.4 ± 17.8 50 (40–55)	47.5 ± 14.7 50 (40–55)	$p = 0.83$ (MW)
Immotile %	52.6 ± 16.6 50 (45–60)	53.0 ± 18.3 50 (43.8–60)	52.2 ± 14.8 50 (45–60)	$p = 1.0$ (MW)
Normal forms %	8.8 ± 8.0 7 (4-11.3)	9.1 ± 5.5 9 (4.8–12.3)	8.6 ± 9.9 6 (3–10)	$p = 0.015$ * (MW)
Head defects %	63.8 ± 16.7 65 (55–75)	62.3 ± 13.0 62 (54.8–70.5)	65.3 ± 19.8 70.5 (55-79.5)	$p = 0.051$(MW)
Neck and midpiece defects %	14.9 ± 11.3 12 (5–25)	8.1 ± 6.6 6 (3-10.3)	21.9 ± 10.9 25 (14.5–31.5)	$p < 0.0001$* (MW)
Tail defects %	11.9 ± 12.2 6 (2-20.5)	5.0 ± 5.5 2 (1-8.3)	18.9 ± 13.1 21 (5–30)	$p < 0.0001$* (MW)

Table 1. Seminal parameters of total patients and HPV-negative and HPV-positive groups. Data were described by mean ± SD and median(IQR) or percentage. *MW* Mann-Whitney test; Test for Normal distribution was performed by Shapiro-Wilk test. *Significant test ($p < 0.05$).

Multivariate analysis	Coefficient	Standard error	OR	95% CI	*p*-value
Null model vs. full model					< 0.0001*(C)
HPV/rapid progressive motility %	0.09	0.04	1.09	1.02–1.17	0.0148*
HPV/slow progressive motility %	− 0.07	0.03	0.94	0.89–0.99	0.0168*
HPV/non-progressive %	0.01	0.03	1.01	0.96–1.06	0.62
HPV/normal forms %	− 0.02	0.03	0.98	0.92–1.05	0.60
HPV/neck and midpiece defects %	0.11	0.04	1.11	1.04–1.19	0.0026*
HPV/tail defects %.	0.05	0.04	1.06	0.98–1.14	0.14
Constant	− 1.66	0.76	–	–	0.0286*

Table 2. Multiple logistic regression between HPV variable and significant independent variables described in Table 1. *OR* odds ratios; *CI* odds ratios confidence interval at 95%; The null model= $-2\ln[L_0]$, where L_0 was the likelihood of obtaining the observations if the independent variables did not affect the outcome, the full model: $-2\ln[L_0]$, where L_0 was the likelihood of obtaining the observations with all independent variables incorporated in the model; C = chi-square test. *Significant test.

In the HPV-positive group, we found significant negative correlations between DFI and rapid progressive motility (rho=-0.64, $p < 0.0001$) and progressive motility (rho=-0.56, $p < 0.0001$), as well as between non-progressive sperms and rapid progressive motility (rho=-0.27, $p = 0.0349$), and progressive motility (rho=-0.35, $p = 0.0066$), and between immotile sperm rate and rapid progressive motility (rho=-0.73, $p < 0.0001$), slow progressive motility (rho=-0.46, $p = 0.0002$), and progressive motility (rho=-0.86, $p < 0.0001$) (Table 3).

A significant positive correlation was found for sperm concentration/ml and rapid progressive motility (rho = 0.49, $p = 0.0001$), slow progressive motility (rho = 0.41, $p = 0.0012$), and progressive motility (rho = 0.63, $p < 0.0001$), and for normal forms and rapid progressive motility (rho = 0.43, $p = 0.0006$), and progressive motility (rho = 0.49, $p = 0.0001$). Significant positive correlations were found only for the two morphological parameters of neck and midpiece defects and tail defect with rapid progressive motility (rho = 0.28, $p = 0.032$ and rho = 0.38, $p = 0.0025$) (Table 3).

The correlation coefficients of HPV-negative and HPV-positive groups are compared in Table 4. A significant decrease of rho was observed for rapid progressive motility and DFI between HPV-negatives and HPV-positives (-0.30vs-0.64; $p = 0.0161$), i.e. the negative relationship between rapid progressive motility and DFI was higher in HPV-positive patients. Conversely, a significant increase was observed for slow progressive motility and DFI (-0.54vs0.03; $p = 0.0021$*), as the statistical significance of the correlation between DFI and slow progressive

Parameters	rho (*p*-value) Rapid progressive motility %	rho (*p*-value) Slow progressive motility %	rho (*p*-value) Progressive motility%
Negative for HPV infection, 61 ptz			
Sperm DFI %	− 0.30 (0.0182*)	− 0.54 (< 0.0001*)	− 0.51 (< 0.0001*)
Sperm concentration/ml	0.42 (0.0008*)	0.55 (< 0.0001*)	0.54 (< 0.0001*)
Non progressive %	− 0.18 (0.15)	0.02 (0.89)	− 0.12 (0.34)
Immotile %	− 0.53 (< 0.0001*)	− 0.58 (< 0.0001*)	− 0.66 (< 0.0001*)
Normal forms %	0.27 (0.0359*)	0.49 (0.0001*)	0.44 (0.0003*)
Head defects %	0.17 (0.41)	− 0.06 (0.63)	0.01 (0.95)
Neck and midpiece defects %	0.26 (0.11)	0.22 (0.40)	0.04 (0.77)
Tail defects %	− 0.20 (0.13)	0.14 (0.28)	− 0.01 (0.92)
Positive to HPV infection, 60 ptz			
Sperm DFI %	− 0.64 (< 0.0001*)	0.03 (0.79)	− 0.56 (< 0.0001*)
Sperm concentration/ml	0.49 (0.0001*)	0.41 (0.0012*)	0.63 (< 0.0001*)
Non progressive %	− 0.27 (0.0349*)	− 0.13 (0.33)	− 0.35 (0.0066*)
Immotile%	− 0.73 (< 0.0001*)	− 0.46 (0.0002*)	− 0.86 (< 0.0001*)
Normal forms %	0.43 (0.0006*)	0.22 (0.09)	0.49 (0.0001*)
Head defects %	0.10 (0.43)	− 0.21 (0.10)	− 0.09 (0.51)
Neck and midpiece defects %	0.28 (0.032*)	− 0.15 (0.24)	0.13 (0.33)
Tail defects %	0.38 (0.0025*)	− 0.18 (0.17)	0.17 (0.21)

Table 3. Degree of association of infection using Spearman's rho correlation coefficient between rapid progressive motility, slow progressive motility, progressive motility and all other parameters in Table 1, with our sample stratified into HPV negative and positive patients. *Significant test ($p < 0.05$).

Comparison of correlation coefficients	HPV-*N* (61 ptz) vs. HPV-*P* (60 ptz) rho1 vs. rho2 (*p*-value)
RPM%/Sperm DFI %	− 0.30 vs. − 0.64 (0.0161*)
RPM%/Sperm concentration/ml	0.42 vs. 0.49 (0.64)
RPM%/Non progressive %	− 0.18 vs. − 0.27 (0.61)
RPM%/Immotile%	− 0.53 vs. − 0.73 (0.07)
RPM%/Normal forms %	0.27 vs. 0.43 (0.33)
RPM%/Neck and midpiece defects %	0.26 vs. 0.28 (0.91)
RPM%/Tail defects %	− 0.20 vs. 0.28 (0.29)
SPM%/Sperm DFI %	− 0.54 vs. 0.03 (0.0021*)
SPM%/Sperm concentration/ml	0.55 vs. 0.41 (0.33)
SPM%/Immotile %	− 0.58 vs. − 0.46 (0.38)
SPM%/Normal forms %	0.49 vs. 0,22 (0.09)
PM%/Sperm DFI %	− 0.51 vs. − 0.56 (0.71)
PM%/Sperm concentration/ml	0.54 vs. 0.63 (0.46)
PM%/Non progressive %	− 0.12 vs. − 0.35 (0.19)
PM%/Immotile %	− 0.66 vs. − 0.86 (0.0073*)
PM%/Normal forms %	0.44 vs. 0.49 (0.73)

Table 4. Comparison of correlation coefficients of Table 3 between HPV positive and negative patients. *Significant test, *RPM*% rapid progressive motility %, *SPM*% slow progressive motility %, *PM*% progressive motility (%), *HPV-N* HPV-negative, *HPV-P* HPV-positive.

motility was lost in HPV-positive samples. Finally, a significant decrease was observed for rho values of progressive motility and immotile sperm rate (-0.66vs-0.86; $p = 0.0073$), i.e., the negative relationship between progressive motility and immotile sperm rate increased in HPV-positive patients. A graphic representation of the correlations between DFI and slow, rapid, and progressive motility for HPV- negative and positive groups are shown in Figs. 1 and 2.

Furthermore, we compared and evaluated the seminal parameters in HPV-positive samples divided into positive to lr and hr genotypes (Table 5).

In this case, a significantly lower rate of DFI (median: 19.6vs30.4, $p = 0.0083$), immotile (median: 45vs52.5, $p = 0.0393$) and head defects (median: 59vs73,5, $p = 0.028$) was found in lr-positives than in hr-positive, while

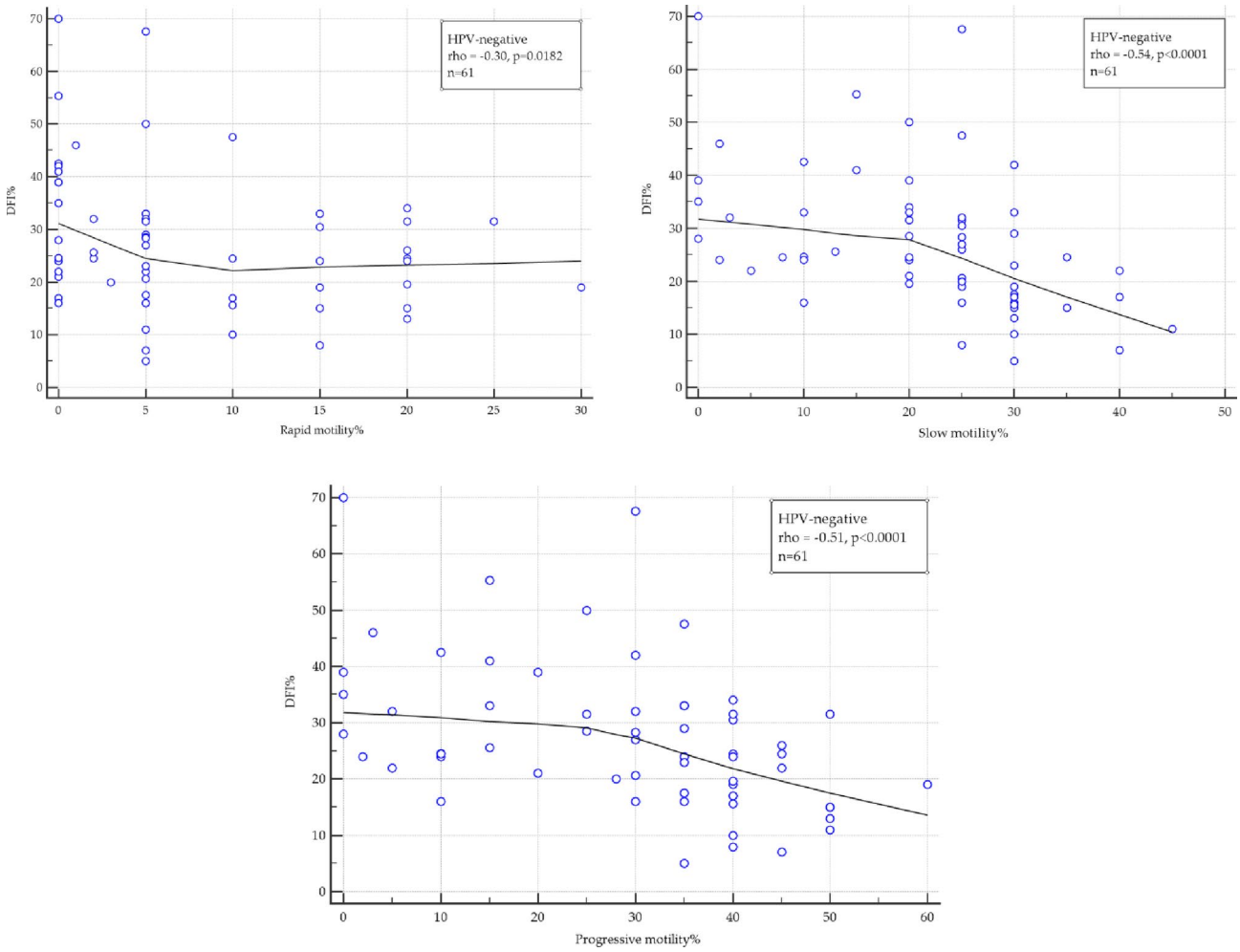

Fig. 1. Scatter plots of the correlations between sperm DFI% and rapid progressive motility, slow progressive motility, and progressive motility in patients without HPV infection.

an opposite result was found for total motility (median: 55vs47.5, $p=0.0435$) and normal forms (median: 9vs5, $p=0.0367$).

In Table 6 we reported the multiple logistic regression analysis between the HPV risk variable, defined as a dichotomous variable (0 = low risk, 1 = hrHPV or presence of both lr and hr genotypes) and the significant variables of Table 3, namely, DFI, total motility, immotile sperms, normal forms, and head defects. Of all significant associations found in Table 3, only DFI was identified as a significant positive predictor of the HPV risk variable (OR = 1.09, $p=0.0262$).

Discussion

From 1990 to 2019, the global prevalence of male infertility has seen an increase of 76.9%, with a steady decline in semen quality[30]. This alarming trend can be attributed to several causes, including environmental factors, unhealthy habits, and diseases such as reproductive tract infections[31]. However, due to inconsistent experimental results, the link between certain conditions and infertility remains doubtful.

Within this framework, the WHO[2] published new guidelines for the execution and interpretation of semen analysis, in which DFI, slow progressive motility and rapid progressive motility have been included as new parameters for evaluating semen quality[32]. In particular, until 2010, sperm motility was assessed using four different parameters: rapid progressive motility, slow progressive motility, non-progressive and immotile spermatozoa. From 2010 to 2021, this distinction was partially abandoned, with rapid and slow progressive motility being combined into a single "progressive motility". However, the latest edition of the WHO guidelines has reintroduced the distinction between the two parameters. It can be assumed that considering this update in sperm evaluation, new and clearer insights will be gained on controversial topics such as the correlation between HPV infection and male infertility.

With this in mind, we performed a seminal analysis on 121 sperm samples, divided into HPV-negative and HPV-positive, following the WHO 2021 guidelines and focusing on the distinction between slow and rapid progressive motility. Therefore, unlike our last paper[22], where we based our results on total motility according to

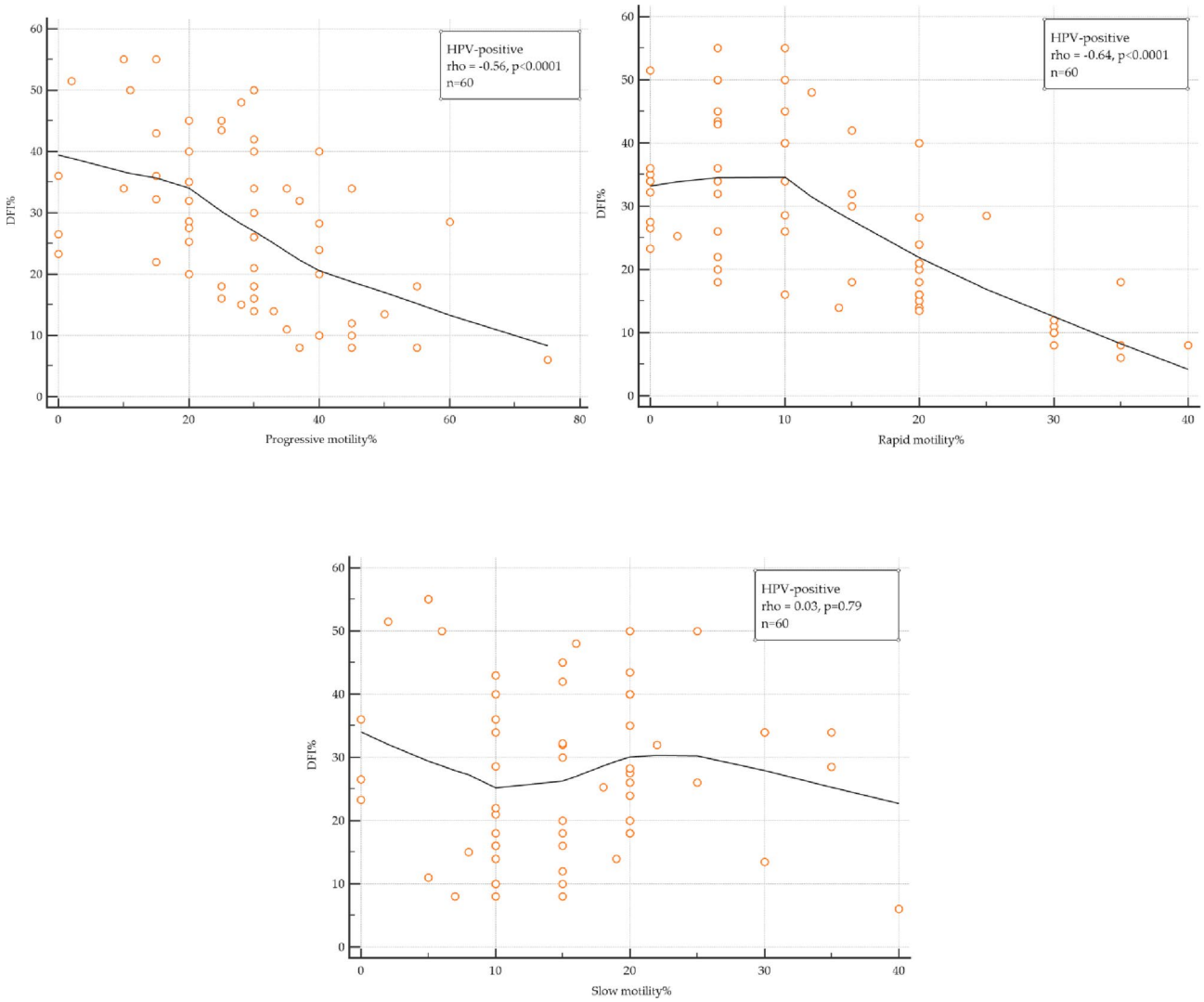

Fig. 2. Scatter plots of the correlations between sperm DFI% and rapid progressive motility, slow progressive motility, and progressive motility in patients with HPV infection.

the WHO 2010 guidelines, here we have a more detailed analysis of how HPV infection affects sperm motility not potentially flattened by the use of the sole total progressive motility parameter.

The overall HPV prevalence of 49.6% in the study cohort is quite high in comparison with the prevalence reported in the literature for infertile men[27]. This discrepancy can be easily explained by the characteristics of the enrolled population, which consisted entirely of male partners of HPV-positive women.

Otherwise, the high proportion of hrHPV aligns with the results of other studies, in which hrHPVs are the most represented in the infertile male population[27].

All the studies focusing on the correlation between HPV and sperm motility do not refer to the WHO 2021 classification, evaluating only total progressive motility. It could be hypothesised that the distinction between slow and rapid progressive motility could bring out differences between HPV-positive and negative samples that would not have been revealed with the evaluation of the sole progressive motility. Indeed, in this study, while the comparison of the two groups' total progressive motility values did not produce statistically significant results, the rates of slow and rapid progressive motility were significantly different depending on HPV positivity, with slow spermatozoa more represented in the HPV-negative group and rapid progressive sperms more numerous in HPV-positive samples.

This finding, although unexpected and counterintuitive, as it would suggest a higher performance of HPV-positive spermatozoa, finds some consistency with two studies by Connelly et al.[33] and Brossfield et al.[34]. The two papers describe a similar approach consisting of the evaluation of semen parameters after exposure of washed sperm cells to HPV-DNA, specifically E6-E7 fragments[33] and L1 fragments[34]. The analysis of the sperm status revealed an increase in motility and progressive motility, respectively. Even though the experimental condition described in these two papers differs from an ongoing infection, such a peculiar correlation may be worthy of attention. Moreover, talking about the molecular mechanism behind this correlation, an interesting clue comes from Cruz-Gregorio et al.[35] paper, which suggested that E6 oncoprotein from HPV16 and − 18 promotes

Parameters	Low risk	High risk	*p*-value (test)
Patients	14	46	
Sperm DFI %	19.6 ± 8.5 19 (12-27.5)	30.4 ± 14.0 32 (18–42)	*p* = 0.0083*(T)
Sperm concentration/ml	46.6 ± 46.8 23.75 (16–55)	40.6 ± 40.4 29 (15–53)	*p* = 0.86(MW)
Rapid progressive motility %	18.5 ± 10.7 20(14–30)	12.0 ± 10.8 10(5–20)	*p* = 0.051(MW)
Slow progressive motility %	14.9 ± 9.3 12.5(10–20)	15.5 ± 8.5 15(10–20)	*p* = 0.59(MW)
Progressive motility %	33.4 ± 13.4 34 (30–40)	27.6 ± 15.0 29 (20–35)	*p* = 0.20(T)
Non-progressive%	18.2 ± 7.7 15 (15–25)	18.7 ± 7.2 20 (14–25)	*p* = 0.87(MW)
Total motility %	51.6 ± 15.4 55 (50–60)	46.2 ± 14.4 47.5 (40–55)	*p* = 0.0435*(MW)
Immotile %	47.7 ± 16.0 45 (40–50)	53.5 ± 14.3 52.5 (45–60)	*p* = 0.0393*(MW)
Normal forms %	14.1 ± 13.6 9 (5–25)	6.8 ± 7.9 5 (2–9)	*p* = 0.0367*(MW)
Head defects %	54.3 ± 22.4 59 (29–75)	68.6 ± 17.8 73.5 (55–84)	*p* = 0.028*(MW)
Neck and midpiece defects %	21.6 ± 9.1 20 (15–25)	22.0 ± 11.5 25 (10–32)	*p* = 0.55(MW)
Tail defects %	17.4 ± 12.2 17 (5–30)	19.4 ± 13.5 23.5 (5–30)	*p* = 0.70(MW)

Table 5. Seminal parameters of HPV-positive patients stratified in low and high risk. *MW* Mann-Whitney test; T = t-Student test; test for Normal distribution was performed by Shapiro-Wilk test; Data were described by mean ± SD, median (IQR) or percentage. *Significant test ($p < 0.05$).

Multivariate analysis	Coefficient	Standard error	OR	95% CI	*p*-value
Null model vs. full model					0.0145* (C)
Risk/DFI %	0.09	0.04	1.09	1.01–1.19	0.0262*
HPV/total motility %	0.04	0.13	1.04	0.80–1.34	0.78
HPV/immotile %	0.04	0.13	1.04	0.81–1.33	0.78
HPV/normal forms %	0.02	0.06	1.02	0.90–1.14	0.78
HPV/head defects %	0.05	0.03	1.05	0.99–1.12	0.12
Constant	− 7.9	12.8	–	–	0.53

Table 6. Multiple logistic regression between risk variable and significant independent variables described in Table 5. *OR* odds ratios; *CI* odds ratios confidence interval at 95%; The null model = $-2\ln[L_0]$, where L_0 was the likelihood of obtaining the observations if the independent variables did not affect the outcome, the full model: $-2\ln[L_0]$, where L_0 was the likelihood of obtaining the observations with all independent variables incorporated in the model; C = chi-square test. *Significant test.

mitochondrial metabolism and cellular respiration in a head and neck cancer cell model. This finding was coupled with no significant changes in ATP-linked respiration and a detection of higher ROS concentrations, leading to oxidative stress.

As concerns sperm morphology, HPV-positivity appears to be correlated with substantial morphological abnormalities, involving with the highest statistical significance the neck and the midpiece, as emerged by our multivariate analysis. These findings join numerous studies already describing an HPV-induced deterioration of overall sperm morphology[13,15,36–38]. Furthermore, in HPV-positive samples, correlation analysis between morphological defects of the neck and midpiece and rapid progressive motility revealed a highly statistically significant positive correlation. In other words, the greater the morphological deterioration of the neck and midpiece, the greater the rapid progressive motility.

Sperm genomic DNA integrity is a crucial factor influencing reproductive potential, with DFI higher than 30% being associated with male infertility, recurrent pregnancy loss and poor ART outcomes[39]. One of the main causes of sperm DNA fragmentation has been identified as a high level of oxidative stress, which can be induced by several environmental and pathological factors. These include ageing, obesity, diet, smoking, radiation, toxins, pollutants, testicular disorders such as varicocele, and genital tract infections, such as sexually transmitted ones[39]. With the publication of the 6th edition of the WHO Laboratory Manual for the Examination

and Processing of Human Semen, greater attention has been paid to the role of sperm DNA fragmentation, which is now included among the parameters evaluating sperm quality.

The effect of HPV infection on sperm DNA integrity is controversial, as the results of the few studies that have evaluated it are not entirely consistent[18,27–29,33].

In this study, even if no difference was found in the DFI of HPV-positive and negative samples, once again only the separate count of rapid and slow spermatozoa allowed us to point out significant correlation ratios. In fact, in both HPV-positive and HPV-negative, DFI is negatively correlated with rapid and total progressive motility, but only negatives have the same association with slow progressive motility. The results suggest how an impairment of sperm genomic integrity could affect reproductive health through a consistent impairment of spermatozoa motility.

Recently, Perez-Soto et al.[38] described an association between hrHPV infection, high levels of oxidative stress and sperm cell impairment. Indeed, their findings highlighted that HPV-positivity was correlated with an overexpression of the cytochrome P450 2E1 gene, related in turn to an elevated ROS production and an oxidation-induced damage of polyunsaturated fatty acids, of which spermatozoa plasma membrane is rich. They pointed out how genomic impairment correlated with morphological defects, among which tail defects were associated with high ROS levels. They hypothesized that defects affecting sperm midpiece could impair mitochondria activity, consecutively leading to high levels of oxidative stress levels and morphological anomalies.

Other studies[40] on the molecular mechanism of HPV infection suggest that E6 oncoprotein from hrHPV16 and − 18 increases mitochondrial metabolism in squamous cell carcinoma and indirectly promotes cell respiration and the expression of mitochondrial ATP-synthetase through the production of ROS, and finally causes DNA damage. It also described an isoform of hrHPV-16 E6* that enhances mitochondrial dysfunction, oxidative stress and DNA damage but does not induce apoptosis because it decreases p53 levels. In addition, Gregorio et al. reported, in cervical cancer cells, that overexpression of E2 interacts with mitochondrial complexes III and IV and produces an alteration in the mitochondrial membrane potential, the loss of mitochondrial cristae, a consequent loss of mitochondrial morphology and apoptosis into the mitochondria. In addition, the E4 protein from hrHPV-16 binds both cytokeratin and mitochondria and provokes the release of mitochondria from microtubules.

A link between HPV and oxidative stress was also proposed by Pellavio et al.[41], which showed the colocalization, confirmed by a 3D simulation model, of HPV and Aquaporins-8 (AQP8), involved in ROS elimination and found precisely in the midpiece of sperm cells and seemingly in mitochondrial membranes. These findings were complemented by the detection of a reduced sperm osmotic permeability, found even in normozoospermic males, that would indicate an actual alteration of canal functionality. This idea partly matches with the study by Kato et al.[13], in which HPV virions have been localized not only in the head but also in the midpiece of spermatozoa, where mitochondria are placed.

Although these preliminary observations can only be speculative, it may not be a coincidence that the morphological anomalies we found affect the neck and the midpiece, where both mitochondria and AQP8 are located. In other words, HPV virions located in the midpiece of spermatozoa could induce both an alteration of morphology and of ACP8 functionality, which would result in a first increase of sperm motility, but gradually in a rise of oxidative stress, affecting negatively reproductive potential.

Although the study we conducted is preliminary and observational, we hypothesized a similar mechanism of action of some HPV genotypes in sperm cells, based on scientific literature and our observation, that could explain the effects of HPV on sperm motility and morphology, as described in supplementary material 1.

HPV could bind spermatozoa via aquaporins, as described by Pellavio et al.[41], and/or other unknown receptors. Different genotypes can bind different parts of sperm cells: head, midpiece or other regions and, probably, different receptors on the sperm's surface, as described by Schillaci et al.[14], Foresta et al.[17], and Kato et al.[13]. The binding between sperm membrane receptor and HPV could induce an intracellular response, that may produce an alteration of functions of aquaporins and/or a Ca2 + release, that promotes rapid progressive motility in the spermatozoa, or other patterns, such as osmotic permeability, that could influence sperm morphology, especially in the tract of neck and midpiece where located mitochondria and aquaporins. Additionally, the expression of early viral genes, such as hrHPV-16 and − 18 E6, as described by Cruz Gregorio et al.[35], could enhance cellular metabolism and ATP-synthetase through the indirect via of ROS production, causing mitochondrial dysfunction. Sperm mitochondria are known to uptake calcium and are possibly the sites of calcium storage, so a mitochondrial dysfunction could also promote the release of calcium[42].

This mechanism could explain both the enhancement of rapid progressive motile sperm and higher levels of DFI in infected sperms. However, a different isoform of early protein could differently act. For example, the isoform E6* could enhance mitochondrial metabolism without an increment of DFI, for reducing p53 levels. The presence of isoforms with different activity could partially explain conflicting data on HPV and DFI levels reported in literature[23]. So, it is possible that the differences in DFI levels of HPV-infected samples could be due to different activities of isoforms on levels of p53.

Differently from somatic cells, spermatozoa are fully differentiated germ cells with a highly organized and complex ultrastructure and the interaction of viral proteins with their cytoskeleton warrants further investigation. While K. Raj et al.[43] have demonstrated the binding of hrHPV-16 E1 and E4 proteins to the cytokeratin network in epithelial tissues, leading to mitochondrial detachment from microtubules, the situation in human spermatozoa is less understood.

A. L. Kierszenbaum[44] has reported the presence of both epidermis-type and spermatogenetic-type keratins in spermatogenetic cells but the specific binding of mitochondria to these components remains unclear. Mitochondria are ordinately arranged in a unique double-helical structure, called a mitochondrial sheath, that wraps tightly around the axoneme, externally with respect to outer dense fibers (ODFs). M. R. Leung et al.[45]

have provided evidence that the outer mitochondrial membrane directly interacts with either the ODFs or the surrounding cytoskeletal filaments in mammalian.

Based on these observations reported in literature, we hypothesize that the activity of viral proteins could influence not only the loss of mitochondrial cristae but also the bounds between mitochondria and some cytoskeletal proteins, as described by Cruz Gregorio et al.[35], and it could explain the morphological alteration observed in our work, especially in the neck and midpiece regions, where mitochondria are located.

Considering the distinct behaviour of lrHPV and hrHPV genotypes in inducing cellular transformation[46], it seemed necessary to analyse the possible difference between their impact on spermatozoa parameters, a topic rarely addressed in the literature.

In this regard, our results demonstrated that infection with at least one hrHPV most significantly compromises, among other parameters, the integrity of the sperm chromatin.

Similar evidence of a higher DFI being a genotype-dependent impairment has already been described in the literature by Capra et al.[22] and Boeri et al.[18]. This data is also in agreement with Perez-Soto et al.[38], who, as previously mentioned, found in hrHPV infected sperms high levels of ROS, which cause of DNA damage. Although the comparison of lrHPV and hrHPV positive samples did not reveal significant differences in slow and rapid motility parameters, it could be hypothesised that a wider sample could bring out significant differences in rapid progressive motility, whose p-value is particularly close to statistical significance.

The need to better understand how and to what extent HPV infection impacts seminal parameters and male fertility has led to an analysis of semen samples according to the new WHO guidelines. Our findings show how the distinct evaluation of slow and rapid progressive motility can effectively distinguish between HPV-negative and positive samples, with the latter associated with higher rates of rapid progressive motility. Moreover, it appears that HPV-positivity and rapid progressive motility are positively correlated with morphological defects of the sperm midpiece. Although it may seem odd, these findings are congruent with recent studies localizing HPV virions in spermatozoa midpieces, where mitochondria, whose activity is essential in sperm motility, are located.

The small number of examined semen samples represents a limitation to this work and the data presented must be considered with caution. Therefore, in this case, they should be interpreted as preliminary results and should encourage future research.

Methods

Patients, sample collection and sperm parameters analysis

From August 2021 to December 2022, 121 male partners of HPV-positive women attending IVF were received at "D'Arena", Clinical Analysis and Diagnostics Laboratory, Salerno, where they were asked to provide semen samples. Specimens were collected by masturbation after 3–5 days of sexual abstinence. Each patient had to meet the following inclusion criteria: no previous known genital infections; no current infection of C. *trachomatis*, *N. gonorrhoeae*, *U. parvum* and *urealyticum*, *M. hominis*, and *genitalium*; no seropositivity for human immunodeficiency virus type 1 or 2, human T-cell lymphotropic virus type 1 or 2, hepatitis B or C virus, and *T. pallidum*; no genetic disease (e.g. cystic fibrosis), and no inflammatory disorders (e.g.varicoceles).

After liquefaction at room temperature, seminal samples were analysed according to WHO 2021 guidelines for the following parameters: sperm concentration and morphology, non-progressive and immotile sperms, and both slow and rapid progressive motility. Total motility and progressive motility were also analysed. Briefly, after mixing the ejaculate in the original container, an aliquot of 10 µl of semen sample was placed in the Makler's Sperm Counter Chamber (Sefi Medical Instruments), an easy-to-use device for rapid and accurate one-step assessment of sperm concentration and motility from the undiluted sample. Motility was also confirmed by observation of prepared slides containing 10 µl of semen sample covered with a 20×20 mm coverslip using a contrast-phase microscope (Nikon Ci-L) at 20x magnification.

Motility assessment was performed starting with rapidly and slowly progressing spermatozoa to avoid overestimating the number of progressing spermatozoa, and then non-progressing and immotile spermatozoa were counted. Each measurement was confirmed by a separate replicate, approximately 200 spermatozoa were counted per replicate. To assess sperm morphology, each sample was washed and diluted 10-fold to a maximum of 50×106 spermatozoa/ml to avoid overlapping of spermatozoa. An aliquot of 10 µl of the diluted semen was then applied to the slides, blotted, air-dried, fixed and stained according to the DiffQuick rapid staining protocol (MGG quick stain, Bio-Optica, It). Slides were observed with a light microscope (Nikon Ci-L) using a 100x oil immersion objective.

In parallel, one aliquot of semen was sent to the Virology Laboratory of UOC of Microbiology and Virology, Polyclinic Hospital, Palermo, Italy, where HPV detection was performed, while a second one was sent to the "Check Up" PolyDiagnostics and Research Centre, Salerno, for DNA fragmentation analysis. The study was performed according to The Code of Ethics of the World Medical Association (Declaration of Helsinki), informed consent was obtained from all subjects., and the local Ethics Committee approved the protocol (Project identification code: B71J11000160007).

No financial compensation was provided to the participants.

DNA extraction, HPV detection and genotyping

After liquefaction at room temperature, 200µL of each semen sample was subjected to DNA extraction using the Qiamp DNA mini kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

Detection and genotyping of HPV-DNA were performed using Ampliquality HPV-Type Express v3.0 (AB Analitica, Padua, Italy), which consists of a PCR amplification using biotinylated primers followed by a reverse dot blot hybridisation assay. The technique allows for the identification of 40 HPV genotypes, distinguished into hr and lrHPV according to the International Agency for Research on Cancer (IARC) classification. Specifically,

the assay can detect twenty-two hrHPVs (HPV16, HPV18, HPV26, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV53, HPV56, HPV58, HPV59, HPV64, HPV66, HPV67, HPV68, HPV69, HPV70, HPV73, HPV82) and eighteen lrHPVs (HPV6, HPV11, HPV40, HPV42, HPV43, HPV44, HPV54, HPV55, HPV61, HPV62, HPV71, HPV72, HPV81, HPV83, HPV84, HPV87, HPV89, HPV90).

Sperm DNA fragmentation analysis by SCD test and DFI evaluation

Evaluation of sperm DNA status was carried out through the sperm chromatin dispersion (SCD) technique, as described by Fernandez et al.[47] and Bosco et al.[31]. This approach is based on a controlled DNA denaturation process, which allows the removal of spermatozoa proteins and the subsequent formation of DNA loops, called DNA dispersion halos. The presence and the size of sperm halos indicate DNA status: big and medium halos identify not fragmented genomes, while small, absent halos, and degraded sperms, are classified as fragmented DNA.

Samples at a concentration of $5–10 \times 10^6$/mL needed for preventing sperm DNA halos from overlapping, were processed with a Halosperm® kit (Halotech DNA SL Spain). Then, 500 sperms for each sample were observed using a brightfield microscope (Nikon Ci-L) connected to a Gigabit Camera (Basler Ace ACA780-75GC), which allowed the acquisition of images. After identifying sperm halos, DFI was calculated as the percentage of fragmented nuclei compared to total cells.

Statistical analysis

Data are presented as numbers and percentages for categorical variables and continuous data are expressed as mean ± standard deviation (SD), and median and interquartile interval (IQR= [Q1; Q3]).

Test for Normal distribution was performed by the Shapiro–Wilk test. A T-test was used to compare the mean of unpaired samples. When the distribution of samples was not normal, the Mann-Whitney test was used. Differences between groups were analysed using the chi-square test or Fisher's exact test for categorical variables.

Multiple logistic regression was used to find the best-fitting model to describe the relationship between the dichotomous characteristic of interest (dependent variable) and a set of independent variables.

The degree of association between two non-normal variables was computed using the Spearman correlation coefficient rho. All tests with $p < 0.05$ were considered significant. All data were analysed with Matlab statistical toolbox version 2008 (MathWorks, Natick, MA, USA) for 32-bits Windows.

Data availability

The data supporting the findings of this study are provided within the manuscript and are available on request from the corresponding author, Giuseppina Capra.

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Author contributions

Author contribution: Conceptualization: L.B., G.C., T.N.; Methodology: L.B., G.C., N.S., T.N., M.B.; Software: N.S.; Formal analysis: N.S.; Investigation: T.N., M.B., A.S., G.R.; Resources: G.C., T.N.; Data Curation: N.S., L.B., G.C., M.B.; Writing – Original Draft: M.B., A.S., T.N.; Writing - Review & Editing: L.B., G.C., T.N., N.S.; Visualization: M.B., A.S., N.S.; Supervision: L.B., G.C.; Project administration: L.B., G.C.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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