Original Article

New Approaches to Define The Functional Competency of Human Sperm Subpopulations and Its Relationship to Semen Quality

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Abstract

Background: This study aimed at comparing a comprehensive set of functional and structural sperm characteristics between sperm motility fractions and correlating results to the standard semen parameters. By grouping related variables, our objective was to establish the predictive power of semen parameters and whether they accurately reflect the functionality of sperm motility fractions or merely a small set of parameters within individual fractions.

Materials and Methods: In this non-invasive experimental study, donor semen samples (n=55) were separated via double density gradient centrifugation, isolating a high (HM) and low motile (LM) sperm fraction. Fractions were evaluated for percentage vitality, chromatin integrity, mature spermatozoa, motility and kinematic parameters, hyperactivation, positive reactive oxygen species, intact mitochondrial membrane potential (MMP) and acrosome reaction.

Results: HM fractions had significantly ($P<0.001$) enhanced percentages of induced acrosome reaction (HM, 55.6 ± 14.3%, LM, 25.0 ± 16.5%), motility and kinematic parameters, hyperactivation, vitality (HM, 70.4 ± 9.7%, LM, 47.9 ± 10.3%), mitochondrial membrane intactness (HM, 67.2 ± 10.4%, LM, 44.7 ± 15.0%) and mature spermatozoa (HM, 83.4 ± 10.0%, LM, 64.6 ± 8.2%) with intact chromatin (HM, 80.5 ± 8.1%, LM, 71.3 ± 8.0%). Various sperm morphology abnormalities correlated with LM fractions' grouped motility parameters (range, 0.46 to 0.51; range -0.4 to -0.75), whereas combined semen traits of total motility, progressive motility, viscosity and mucus penetration (MPT) correlated with HM fractions' grouped motility parameters (range, 0.44 to 0.84).

Conclusion: Collectively, total and progressive motility, viscosity and MPT may represent a reliable grouping of semen parameters for predicting the quality of HM sperm fractions. Separating the same donor semen samples into two significantly diverse motility sperm fractions could be a potential model in mimicking the qualities of fertile and sub-fertile males' sperm populations and used for future research on the improvement of sperm subpopulations from males with different fertility statuses.

Keywords: Computer-Assisted Sperm Analysis, Differential Gradient Centrifugation, Hyperactivation, Reactive Oxygen Species, Sperm Motility Subpopulations

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Introduction

The heterogeneous nature of human semen is well-known with distinct cell populations varying in degrees of maturation, functional quality and fertilizing ability (1). The exact physiological role of subpopulations remains unclear although correlations between the percentage of diverse sperm subpopulations in semen and sperm quality, fertility and the ability to resist cryopreservation damage have been reported (2). Many techniques are employed to separate semen into sperm subpopulations but ideally subpopulations representing spermatozoa of high sperm functionality relating to fertilization success should be identified and isolated - a challenge which still remains in modern andrology (3, 4).

Routine semen evaluations include small subsets of larger, heterogeneous number of spermatozoa from a single ejaculate, thereby inherently creating a large variability which may interfere with accurate evaluations of overall sperm quality (5). Thus, semen analysis may provide suitable information for preliminary evaluations of infertile males, but hardly represent true fertility or functional performance of spermatozoa (6). Complementary structural and functional tests with less inconsistencies relating to fertilization outcome such as evaluation of sperm DNA and chromatin integrity, reactive oxygen species (ROS), mitochondrial membrane potential (MMP), acrosome reaction (AR), cervical mucus penetration, motility parameters (particularly sub-populations such as rapid progressive sperm) and hyperactivation (HA) should therefore be utilized (3, 5, 6).

Various sperm selection techniques have been established based on the differentiation methods for sperm density, membrane surface charge, morphology, motility, membrane integrity and nuclear integrity (3,

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7). Colloid centrifugation is such a technique, whereby a subpopulation of motile spermatozoa with good functional and structural integrity is isolated from the seminal plasma (3, 4, 8). Separated pellets generally comprise of higher numbers of motile and morphologically normal spermatozoa in comparison with lower density fractions (8-10). Additionally, recovered pellets should comprise of more spermatozoa with functional MMP, lower levels of ROS and less spermatozoa with apoptotic and necrotic markers (4, 8, 10). Nevertheless, discontinuous density gradient centrifugation (DGC) has been observed to result in high levels of DNA damage and ROS production (4).

With increased reports of male infertility, and an estimated 10 to 15% of men being affected by idiopathic male infertility at prime reproductive age, alternative approaches for fertility diagnosis including both functional and structural sperm tests are essential (11-13). In-depth, sperm assessment methodologies could assist to elucidate unknown factors affecting male fertility, provide individualized infertility treatments and getting valuable insights required for development of more relevant semen quality tests (11, 14). Additional sorting of ejaculates into subpopulations before evaluation may congruently detailed depth information on functional capabilities of entire ejaculates, further assist in the selection of recovered spermatozoa and seminal dose calculations in assisted reproductive technology (ART) (2, 12, 15). Furthermore, understanding and evaluating the biochemical and molecular mechanisms regulating human sperm functionality, especially in sperm subpopulations may assists clinicians in selecting the most appropriate ART treatment (12).

This study aimed to investigate and compare various functional and structural sperm characteristics between two sperm fractions [high motile (HM) and low motile (LM)] and correlate each fraction's results to the standard semen parameters. Despite differences between HM fractions and semen widely reported in literature (4), our approach was to assess a larger set of parameters for both HM and LM fractions and determine whether groupings of related parameters could refine potential relationships between neat semen and the functional quality of two individual motility fractions. Furthermore, since the same donor semen samples were used to produce the two sperm fractions, this approach could provide a good model for comparing sperm functionality in different sperm fractions, potentially mimicking the qualities in fertile and sub-fertile males' sperm populations.

Materials and Methods

Sample collection and standard semen analysis

In this non-invasive experimental study, 55 human semen samples were obtained from 39 healthy male donors as part of a donor program (Division of Medical Physiology, Department of Biomedical Sciences, Stellenbosch University). Samples were incubated permitting liquefaction (30 - 60 minutes at 37ºC in a 5% CO_2 regulated incubator) and processed as recommended by the World Health Organization (16). Semen volume, pH and viscosity were assessed in addition to several sperm parameters, including total motility, progressive motility, sperm concentration, total number of spermatozoa, mucus penetration (MPT), vitality and morphology (analysed with Sperm Class Analyser® (SCA®) computer-aided sperm analysis (CASA) system, version 6.2; Microptic S.L., Barcelona, Spain). As the study focused on investigating the functionality of two sperm motility fractions of different semen qualities, and determining if semen can accurately predict fertility, minimum cut-off points for percentage total sperm motility of 25% was used. Although this percentage is below the WHO lower reference limit, it was a non-biased reflection of the donors used, that otherwise had good semen parameters (16). This was a non-invasive *in vitro* study using semen from donors for research purposes only, and approved by the ethical boards of the University of the Western Cape (code 13/10/90) and Stellenbosch University (code N14/06/074). The Helsinki Declaration governing research on humans has been adhered to and each human donor provided written consent (17).

Preparation of sperm fractions

Semen samples were separated through DGC into two sperm motility fractions with AllGrad® 90/45% and AllGrad Wash® (Delfran, Johannesburg, South Africa). Semen aliquots (300 µl) were layered on top of equal volumes of the preheated (37ºC) density gradient 90 - 45%, and centrifuged at room temperature (RT) for 20 minutes at 500 *g*. Resultant top seminal plasma coats were discarded and remaining intermediate (less motile spermatozoa, LM fraction) and bottom (highly motile spermatozoa, HM fraction) pellets were separated into individual Eppendorfs. Separated fractions were re-suspended in 300 µl AllGrad Wash® and centrifuged at 500 *g* for 10 minutes. Washed pellets were re-suspended in non-capacitating human tubal fluid (HTF) to final sperm concentrations of 15 - 25×10^6/ml (18). For the purpose of this study, HTF was prepared without the supplementation of human serum albumin (HSA) in order to obtain an accurate functional representation of the two fractions without the interaction or stimulation of proteins.

Viscosity

Using the viscosity evaluation technique described by Rijnders et al. (19), 3 µl semen aliquots were loaded into preheated (37ºC) four-chamber, 20 µm-depth Leja slides (Leja Products B.V., Nieuw Vennep, The Netherlands) and the filling time recorded in seconds. Viscosity in centipoise (cP) was determined by the following equation:

$y=0.34x+1.34$

where y = viscosity in cP and x = filling time in seconds.

Sperm morphology

Semen aliquots (300 µL) were centrifuged in AllGrad

Wash® at RT for 20 minutes at 500 *g* and subsequent pellets re-suspended in HTF. Morphology smears were prepared (15 µL) and dried slides stained with SpermBlue® fixative and stain mixture (Microptic S.L., Barcelona, Spain) as described by Microptic (20). Coverslips were mounted with DPX mounting (Sigma Aldrich, Cape Town, South Africa) and 100 spermatozoa analysed with the Morphology module of the SCA® software using brightfield optics, a Basler ACA 1300-200uc camera, a blue filter and a 60x objective on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa).

Sperm vitality

Following the BrightVit technique as described by Microptic (20) - semen samples and sperm fractions (n=35) were stained in suspension with BrightVit medium (Microptic S.L., Barcelona, Spain) for 10-15 minutes at 37ºC. Vitality smears (20 µl) were prepared and left to air dry before mounting with a coverslip using DPX mounting medium. Stained smears were viewed using the same equipment as described for sperm morphology.

Sperm motility, concentration and mucous penetration

Total sperm motility, progressive motility, concentration and MPT were assessed with the Motility module of the SCA® software and data captured with a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) attached to a Nikon Eclipse 50i microscope with a 10x positive phase contrast objective, a green filter and a heated stage. Preheated (37ºC) four or eight chamber, 20 µm-depth Leja slides were loaded with 2-3 µL of semen or prepared sperm fractions (n=35), and at least two fields with 200 motile spermatozoa analysed at 50 frames per second (f/s).

Percentages sperm motility assessed included total motility, progressive motility, rapid-, medium- and non-progressive motility as well as rapid-, medium- and slow-swimming spermatozoa. Kinematic parameters recorded for the average (overall fraction) and various progressiveness and swimming speed subpopulations included; curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). ALH was measured as half of the width the VCL track and not as the full VCL wave or doubling of riser values (risers' method) as described by Mortimer (21). Kinematic parameter cut-off values for MPT were VAP >25 µm/seconds, STR >80% and 7.5 µm< ALH >2.5 µm and DANCE was calculated as VCL×ALH.

Hyperactivation

Based on a previously established protocol for induced hyperactivation in human spermatozoa, both 2 mM procaine hydrochloride and 5 mM caffeine (Sigma Aldrich, Cape Town, South Africa) supplemented in HTF were used to induce sperm hyperactivation (22). HTF prepared as capacitating (CAP; HTF supplemented with 0.105 g $NaHCO_3$, 1.1915 g HEPES and 0.6 ml NaOH) and non-capacitating medium (HTF) were used as positive and negative controls respectively (18). Applying the flush technique described by Boshoff et al. (23), each chamber of a preheated (37ºC) four chamber Leja slide was loaded with 1 µl sperm preparation (HM or LM sperm fraction suspended in HTF, n=20) and flushed with 2 µl of each of the four preheated media mentioned above. Percentage hyperactivation [using cut-off values: VCL>150 µm/seconds; LIN<50%; ALH>7 µm (3.5 for SCA)] of at least 200 motile spermatozoa was assessed for each sperm fraction using the Motility module of SCA® and equipment as described for sperm motility,, after 5, 15, 30, 45 and 60 minutes of exposure to each medium (21).

Reactive oxygen species

Dihydroethidium (DHE, Molecular Probes, Eugene, OR, USA) was used to detect spermatozoa positive for ROS. Sperm fractions (n=20) were stained in the dark for 15 minutes in suspension (180 µL) with 20 µL of 20 µM DHE at 37ºC. Following incubation, 5-10 µL of suspension was placed on a clean slide with a coverslip, and immediately analysed using a 100x oil immersion objective and triband filter (excitation wavelengths: 457 nm=blue, 530 nm=green and 628 nm=red) on a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa). Percentage of spermatozoa positive for ROS was calculated after manual assessment of at least 100 spermatozoa.

Mitochondrial membrane potential (ΔΨm)

The Mitochondria Staining Kit protocol (CS0390, Sigma Aldrich, Cape Town, South Africa) assessing MMP was optimized for this specific study. Fractions in HTF (n=20) were stained in suspension (1:1) with MMP staining solution (160 µl dH_2O, 40 µl JC-5 buffer and 1 µl frozen MMP 200x stock solution) and incubated in the dark at 37ºC for 20 minutes. Suspensions were subsequently centrifuged at 500 *g* for 5 minutes at 5-7˚C, pellets re-suspended in 200 µl JC-1 buffer (80 µl JC-5 buffer and 320 µl dH_2O) prepared and cooled on ice before use. Suspensions were centrifuged again as described above and pellets re-suspended in remaining 200 µl JC-1 buffer. Single drops of 5-10 µl suspension was placed on a clean slide with a coverslip, and immediately analysed as described for ROS. Percentage intact MMP was manually assessed for at least 100 spermatozoa for each fraction.

Acrosome reaction

Acrosome reaction was determined with the use of the FlouAcro protocol described by Microptic (20). Fractions (n=35) were divided into positive and negative controls and incubated at 37ºC for 3 hours in 1 ml of preheated (37ºC) capacitating media. Negative controls were treated with 10 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cape Town, South Africa) and positive controls with 10 µl of 1 mM Ca-ionophore. Samples were left to incubate for 15 minutes, after which reactions were terminated with 100 µl of 70% ethanol. Two 5 µl drops of each suspension were

placed on clean slides and left to air dry before fixation in 95% ethanol (United Scientific, Cape Town, South Africa) at 4ºC for 30 minutes. Fixed spermatozoa were stained in a dark room for 30-40 minutes with 80 µl of fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Sigma-Aldrich, Cape Town, South Africa) on each drop. Slides were dipped twice in dH_2O to remove excess stain and subsequently counterstained for 7 minutes with 5 µl Hoechst (H33258, Sigma-Aldrich, Cape Town, South Africa), followed by destaining in dH_2O. Acrosome reaction of at least 100 spermatozoa per fraction were manually assessed as described in section 2.8.

Chromatin maturity and fragmentation

Chromatin maturity and fragmentation were determined following the aniline and toluidine blue protocols proposed by Erenpreisa et al. (24) and Erenpreiss et al. (25). For the assessment of chromatin maturity, dried smears were fixed at RT for 30 minutes in 4% formalin and rinsed in dH_2O. Fixed smears were stained for 5 minutes in 5% aniline blue, and excess stain rinsed off in dH_2O. Smears were subsequently stained in 0.5% eosin for 1 minutes, then rinsed in dH2O and left to air dry. For the assessment of chromatin fragmentation, dried slides were fixed in 96% ethanol-acetone (1:1) at 4ºC for 1 hour, then hydrolyzed in 0.1 N HCl at 4ºC for 5 minutes, and finally rinsed in dH_2O. Smears were subsequently stained for 5 minutes in 0.05% toluidine blue at RT, briefly rinsed in dH_2O and left to dry. After mounting with DPX mounting medium and coverslips, slides were viewed using the same equipment as described for sperm morphology. The percentages of spermatozoa with respectively immature chromatin and fragmented chromatin were manually assessed for at least 100 spermatozoa per fraction (n=20).

Statistical analysis

MedCalc statistical software version 14.8.1 (Mariakerke, Gent, Belgium) was used to calculate basic summary statistics and results were expressed as mean ± standard deviation in all the tables. The D'Agostino Pearson test was used to evaluate the distribution of the data, where after the Student's t test or the Mann-Whitney test was used to compare fractions. Where applicable, one-way analysis of variance (ANOVA) for parametric distributions or the Kruskal-Wallis test for non-parametric distributions was used to compare fractions, time points and treatments. Significance was determined at a level of $P<0.05$. Tables and radar plots were constructed with the use of Microsoft Office Excel™ 2016 (Microsoft Corporation, Redmond, Washington, United States). Additional analyses such as correlation coefficients and multivariate visualisations were performed with Statgraphics® Centurion XVII (Statgraphics Technologies, Inc.) to create Star glyphs and Andrews plots. Multivariate graphs provide additional tools for detecting patterns between cases when data sets are too large for standard scatterplots. The star glyph is a representation of each quantitative variable, and the direction and size of the polygon is accordingly scaled to the values of individual selected semen samples. Semen samples with similar characteristics will thus have star glyphs with a similar size and shape. Andrews plots assist in determining small differences between large data sets, thereby highlighting possible differences between cases with similar values. Multiple regression analysis and principal component analysis were further executed with the use of STATISTICA, version 10 (StatSoft Inc.).

Results

Standard semen analysis

Standard semen analysis parameters of the donor semen samples used in this investigation are displayed in Table S1 (See Supplementary Online Information at www.ijfs.ir). Average standard semen analysis parameter values predominantly fell above the lower reference limits as recommended by the WHO laboratory manual; however, with the exclusion of progressive motility (16).

Motility and kinematic parameters

Compared to LM fractions, values for recovered HM fractions were on average four to six times greater for percentage total motility, progressive motility, rapid progressive motility, medium progressive motility, rapid-, medium - and slow - swimming spermatozoa ($P<0.001$, Fig.1A). As seen in Figure 1B, HM fractions displayed higher values for average kinematic parameters in contrast to the LM fractions-with significant differences seen for VCL ($P<0.001$), VSL ($P=0.01$), VAP ($P<0.001$) and ALH ($P<0.001$). Furthermore, HM sperm fractions had significantly higher values for slow progressivity and medium speed group kinematic parameters (Table S2, See Supplementary Online Information at www.ijfs.ir), namely VCL ($P=0.001$), VAP ($P=0.002$) and ALH ($P=0.01$) of the medium speed group and for VAP ($P=0.01$), VSL ($P=0.03$), LIN ($P=0.03$) and WOB ($P=0.03$) of the slow progressivity group. In contrast, LM fractions obtained significantly higher values for medium speed STR ($P=0.01$) compared to the HM fraction.

Figure 2 illustrates star-glyphs of the two separated sperm fractions from individual semen samples. Each individual star-glyph was constructed using 12 kinematic parameters as indicated in the graph key below. While distinct differences between sperm fractions remain evident - star-glyph plots assist in visualizing similarities/differences between fractions of individual semen samples and within a single fraction group. HM sperm fractions illustrate similarities in star-glyph patterns amongst individual semen samples, whereas LM sperm fractions displayed a more heterogeneous pattern, indicating greater variability in kinematic parameter values within LM sperm fractions as compared to HM sperm fractions. Furthermore, Figure 2 displays the variability between fractions prepared from individual ejaculates. For example, substantial differences in the kinematic parameter values can be observed for the two fractions of semen samples 11 (S11), 14 (S14) and 24 (S24). In contrast, some semen samples illustrated kinematic parameter values that

were largely similar between the two sperm fractions, e.g. sample 3 (S3) where both had low values or sample 16 (S16) where both had high values.

Fig.1: Radar plot of the mean sperm motility and average kinematic parameter measurements for comparison of LM (red line) and HM (blue line) sperm fractions (n=35). **A.** Motility parameter comparison of LM (red line) and HM (blue line) sperm fractions. **B.** Comparison of sperm average motility kinematic measurements for LM (red line) and HM (blue line) sperm fractions. ALH; Amplitude of lateral head displacement, BCF; Beat cross frequency, HM; High motile, LIN; Linearity, LM; Low motile, STR; Straightness, VAP; Average path velocity, VCL; Curvilinear velocity, VSL; Straight-line velocity, WOB; Wobble. Values labelled with an asterisk were significantly different between the two sperm fractions for individual parameters (*; P<0.05 and **; P<0.001).

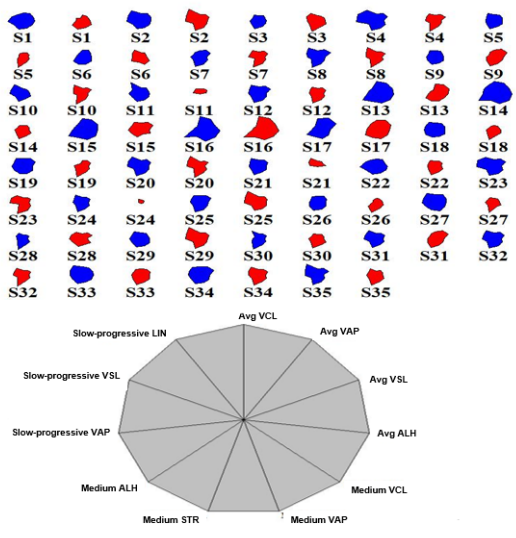

Fig.2: Star-glyph plots for comparison of sperm kinematic characteristics of two fractions separated from individual donor semen samples (n=35) for 12 input parameters (see key above). Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. ALH; Amplitude of lateral head displacement, Avg; Average, LIN; Linearity, S#; Individual semen sample, STR; Straightness, VAP; Average path velocity, VCL; Curvilinear velocity, and VSL; Straight-line velocity.

Hyperactivation

After exposure to 5 mM caffeine, 2 mM procaine, capacitating HTF and non-capacitating HTF medium-significant differences between fractions were presented for each medium and time point, with HM fractions yielding significantly higher mean percentages compared to LM fractions (Fig.3A). No significant difference was apparent among the mediums at individual time points; however, significant differences in percentage sperm hyperactivation were observed when comparing different time intervals for individual mediums. Fractions generally exhibited significant reductions in percentage hyperactivation at 60 minutes compared to 5, 15 and 30 minutes for all the hyperactivation inducing mediums (5 mM caffeine, 2 mM procaine and capacitating HTF). A significant reduction was further observed at 45 minutes compared to 15 minutes for 2 mM procaine in the LM fraction (P=0.003) and 5 mM caffeine in both fractions (LM fraction: P=0.001, HM fraction: P=0.01). In contrast, sperm hyperactivation was significantly higher at 45 minutes compared to 60 minutes for capacitating HTF media in the LM fraction (P=0.002).

Pooled data of all mediums was used to determine the effect of time on percentage hyperactivation for each fraction as illustrated in Figure 3B. Fractions displayed a non-significant increase in percentage hyper activation between the first two time points followed by a steady decrease up to the 60 minutes. However, for HM fractions, the decrease in percentage hyper activation was significant for each time interval from 15 minutes to 60 minutes, whereas for LM fractions the decrease was more gradual. From these results, it is evident that sperm hyper activation should be measured after 15 minutes of exposure to the media.

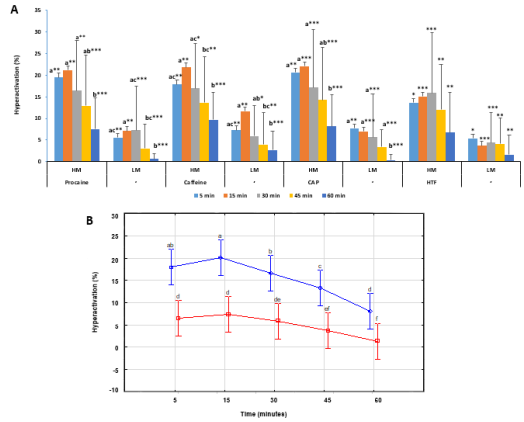

Fig.3: Comparisons of hyperactivation between the LM and HM sperm fractions at different time points and induced with different hyperactivating mediums (n=20). **A.** Bar graph illustrating the mean effect of different hyperactivating mediums (HTF, CAP, procaine and caffeine) on the HM and LM fractions at different time points (5, 15, 30, 45 and 60 minutes). **B.** The effect of time on mean percentage sperm hyperactivation for two sperm fractions separated from individual semen samples. Vertical bars denote SD in Figure 3A and 0.95 confidence intervals in Figure 3B. Time points labelled with different superscript letters (a, b, c, d, e and f) were significantly different (P<0.01) in Figure 3B, whereas time points labelled with different superscript letters (a, b and c) were significantly different (P<0.01) in individual fractions and mediums in Figure 3A. Corresponding bars labelled with an asterisk were significantly different between HM and LM fractions (*; P<0.05, **; P<0.01, ***; P<0.001). CAP; Non-capacitating HTF, HM; High motile, HTF; Human tubal fluid, and LM; Low motile.

Additional structural and functional characteristics

As illustrated in Figure 4A, HM sperm fractions contained significantly greater mean percentages of mature and viable spermatozoa with intact chromatin and MMP and responded significantly better to Ca-ionophore for induced AR (P<0.001). LM sperm fractions had significantly higher mean percentages of spermatozoa containing ROS and spontaneous acrosome reaction (AR-DMSO) - thereby resulting in significantly lower ARIC percentages compared to HM sperm fractions (P<0.001), however still remaining above the abnormal value (ARIC<15%) as recommended by WHO (16).

Figure 4B illustrates an Andrews plot constucted from data of the two fractions of individual semen samples. Each indiviudal line represents a sepecific fraction from a single sample, construscted from the data pertaining to the percentage vitality, ARIC, normal chromatin structure, mature spermatozoa, positive ROS and intact MMP (Table S3, See Supplementary Online Information at www.ijfs.ir). Distinct differences between the HM (blue lines) and LM sperm fractions (red lines) for each semen sample are confirmed in Figure 4B by clear separations of the blue and red lines (label A), thereby illustating the presence of the two distinct groups (subpopulations). In other areas of the plot (label B), the red and blue lines are dispersed from one another and lacking uniformity. This is an indication of how individual data for certain parameters varied among sample fractions; however, the LM sperm fraction (red lines) display larger variation in results compared to the HM sperm fraction (blue lines).

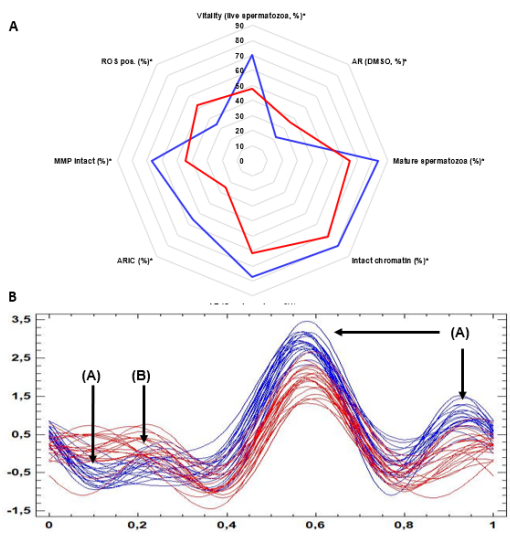

Fig.4: Comparison of the structural and functional characteristics between the HM (blue line) and LM (red line) sperm fractions. **A.** Radar plot comparing HM (blue line) and LM (red line) sperm fractions mean percentage for AR (n=35), viable (n=35) and mature spermatozoa (n=20), spermatozoa with normal chromatin (n=20), positive for ROS (n=20) and intact MMP (n=20). **B.** Andrews plot for comparison of sperm structural and functional characteristics of two fractions separated from individual donor semen samples and constructed using six input parameters (Table S3): percentage vitality, percentage AR, mature spermatozoa, normal chromatin, positive ROS and intact MMP. Data for each parameter was scaled by subtracting its minimum value amongst all the cases and dividing by the range. Values labelled with an asterisk were significantly different between the two sperm fractions for individual parameters (*; P<0.05). AR; Acrosome reaction, ARIC; Acrosome reaction to Ca-ionophore challenge, DMSO; Dimethyl sulfoxide, HM; High motile, LM; Low motile, MMP; Mitochondrial membrane potential, and ROS; Reactive oxygen species.

Correlation and multiple regression analysis of semen characteristics with two sperm motility fractions

Functional and structural parameters of the two sperm motility fractions were correlated with its initial semen parameters as shown in Table 1. Fraction parameters were arranged into two main groups based on the sperm motility and structure. Sub-groups for sperm motility comprised of the motility and kinematics for different speed groups (average, rapid, medium and slow) in addition to hyperactivation, whereas sperm structure comprised of percentage intact MMP, positive ROS, ARIC and immature spermatozoa. Kinematic parameters were further grouped into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) categories. Hyperactivation included both control (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF) percentages. It should be noted that correlations were calculated for 20 semen parameters and 96 sperm fraction parameters, but that data presented in Table 1 only indicate significant (P<0.05) correlations where r≥0.40.

Semen parameters appeared to correlate more with the HM sperm fractions' grouped motility parameters, whereas correlations between the semen and LM sperm fraction parameters appeared to be equally dispersed in both motile and structural grouped parameters. Furthermore, majority of the sperm morphology parameters seemed to largely correlate (mostly negative) with the LM fractions' parameters, whereas semen viscosity appeared to correlate (majority positive) with majority of the HM fractions' parameters. Taking all correlations into account, it seems that a positive relationship exists among total sperm motility, viscosity of semen and fractionated sperm with high motility and swimming speeds in addition to hyperactivation capabilities. On the other hand, sperm morphology defects in semen samples were indicative of sperm fractions with less motile and immature sperm, less potential for hyper activation and acrosome reaction, lower MMP and more ROS.

Illustrated in Table 2 is the R-squared (R2) and beta coefficients (b^*) from the multiple regression analysis used to determine the relationship between four groups of semen parameters (20 predictor variables) and the HM sperm fraction's motility and structural parameters (96 dependent variables). Group I comprised of semen concentration and volume, group II of total motility, semen viscosity, MPT and progressive motility, group III of semen vitality, normal chromatin integrity and mature spermatozoa, and Group IV of semen morphology parameters which were further split into two sub-groups due to multicollinearity (inter-correlations amongst the independent variables which could result in a disturbance of the data) between parameters. Motility and structural parameters of the HM fraction were further grouped and categorised as previously mentioned in Table 1. Data from semen groups with significant (P<0.05) predictor variables and beta coefficients with $b^* \geq 0.40$ were presented in Table 2. Due to incomplete cases in results of the LM fraction, multiple regression analysis was only preformed on the HM sperm fraction.

Table 1: Significant (P<0.05) correlations (r≥0.40) between conventional standard semen parameters of whole semen and sperm structural and functional arameters of density-gradient centrifugation separated sperm fractions (high motile and low motile)

	Motility grouped parameters																		Structure grouped parameters		
	Motility sub-group					Kinematics sub-group									HA sub-group						
							Avg		Rapid			Medium			Slow						
Semen characteristics and fraction	Total	Progressive	Non-progressive	Rapid	Medium	Velocity	Linear	Vigour	Velocity	Vigour	Velocity	Linear	Vigour	Velocity	HTF	Induced	MMP	ROS	ARIC	Immature
Volume																				
HM																				
LM					-0.46															
pH																				
HM																			-0.49	
LM																				
Conc.																				
HM												0.41				-0.49				
LM																				
MPT																				
HM																-0.49				
LM				-0.43																
Visc																				
HM	0.47	0.69	-0.43	0.69		0.61		0.54	0.51	0.52	0.58	-0.45	0.55		0.53	0.75				
LM				0.43					0.62	0.57						0.48	0.61		-0.47	
Tot. mot																				
HM															0.53	0.50				-0.60
LM																			-0.42	
Prog mot																				
HM																				
LM										0.55										
Normal morph																				
HM																				
LM			-0.40			0.46	0.51							0.40			-0.46			
Normal acro																				
HM															0.45					
LM																0.50				
Head defects																				
HM																0.49				
LM														-0.46			0.52			
Midpiece defects																				
HM												0.41								
LM																				
Tail defects																				
HM																				
LM					0.44										-0.53					
Cytopl droplets										-0.64						-0.75			0.44	
HM																				
LM												0.41		-0.40						
TZI	-0.45			-0.40				-0.40								-0.52		0.52		
HM																				
LM																				
DI	-0.43	-0.45		-0.40		-0.42		-0.40							-0.63	-0.51				0.49
HM																				
LM																				
MAI							-0.40							-0.42						
HM																				
LM																-0.48				
							-0.41													

ARIC; Acrosome reaction to Ca-ionophore challenge, Avg; Average, Conc; Concentration, Cytopl droplets; Cytoplasmic droplets, DI; Deformity index, HA; Hyperactivation, HM; High motile fraction, HTF; Human tubal fluid, LM; Low motile fraction, MAI; Multiple abnormalities index, MMP; Intact mitochondrial membrane potential, MPT; Mucous penetration test, Normal acro; Normal acrosome, Normal morph; Normal morphology, Prog mot; Progressive motility, ROS; Reactive oxygen species, Tot. mot; Total motility, TZI; Tetrazoospermic index, VCL; Curvilinear velocity, Visc; Viscosity, and VSL; Sraight-line velocity.

Table 2: Significant (P <0.05 and P <0.01) beta coefficients (b*) ≤0.40 and R-squared (R2) values from the multiple linear regression analysis used to determine the relationship between four groups of semen parameter parameters (20 predictor variables) and the HM sperm fraction's motility and structural grouped parameters (96 dependent variables)

Basic semen parameter groups			Motility grouped parameters									Structure grouped parameters		
			Motility sub-group							HA sub-group				
			Total	Prog	Nprog	Rapid	Medium	Slow	Rprog	HTF	Induced	IM	Mature	MMP
Group I	R										0.53			
	R2										0.28			
	b*	Conc									-0.47			
		Vol												
Group II	R		0.60	0.65	0.51	0.74	0.60		0.62	0.68	0.83	0.71	0.75	
	R2		0.36**	0.43**	0.26	0.55**	0.36**		0.39**	0.46*	0.69**	0.5*	0.57**	
	b*	TM										-0.64	0.69	
		Visc	0.48	0.67	-0.52	0.76	-0.57		0.60	0.98	1.14			
		MP												
		PM								-0.62	-0.69			
Group IV (A)	R		0.62				0.68				0.85			0.74
	R2		0.38				0.46*				0.72*			0.54
	b*	Head def					0.42				0.97			
		Midpiece def					0.62							
		Tail def	-0.42				0.44							
		Cytopl droplets												
		MAI					-0.57							
		Micro												
		Macro												1.08
		Norm acro	-0.56								0.78			
Group IV (B)	R			0.54		0.53	0.67	0.58			0.82			
	R2			0.30		0.28	0.45*	0.33			0.68*			
	b*	Norm. morph					-0.76							
		Tail def						-0.52						
		Cytopl droplets									0.47			
		TZI		0.73		-0.54	0.51	0.69			-0.99			
		DI		-0.82			-0.69							
		Micro												
		Macro												
		Norm acro												

Table 2: Continued

Basic semen parameter groups			Kinematics sub-group															
			Avg			Rapid		Medium			Slow			Rap prog			Mprog	Sprog
			Velocity	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Velocity	Linear	Vigour	Vigour	Linear
Group I	R																	
	R2																	
	b*	Conc																
		Vol																
Group II	R		0.84	0.80	0.78	0.49	0.68	0.81	0.64	0.80	0.45	0.45	0.57	0.80		0.73		0.44
	R2		0.70**	0.65**	0.62**	0.24	0.46**	0.66**	0.41**	0.65**	0.20	0.20	0.33*	0.63**		0.53**		0.19
	b*	TM				-0.45												
		Visc	0.87	0.83	0.84		0.68	0.84	-0.51	0.80	-0.44	-0.42	-0.50	0.82		0.72		-0.43
		MP																
		PM																
Group IV (A)	R			0.44		0.55				0.57					0.51			
	R2			0.19		0.30				0.32					0.26			
	b*	Head def																
		Midpiece def								0.53								
		Tail def		-0.48														
		Cytopl droplets																
		MAI													0.78			
		Micro																
		Macro																
		Norm acro				0.48												
Group IV (B)	R			0.66		0.61	0.55										0.57	
	R2			0.43*		0.37	0.30										0.33	
	b*	Norm. morph		-0.60													-0.70	
		Tail def																
		Cytopl droplets																
		TZI																
		DI																
		Micro				-0.64												
		Macro				-0.74												
		Norm acro		0.45		0.54	0.48											

Semen parameter groups comprised of: (group I) - concentration and volume; (group II) - total motility, semen viscosity, mucus penetration and progressive motility and (group IV) - semen morphology parameters, which were further split into two sub-groups [groups IV (A) and (B)] due to multicollinearity between parameters. HM sperm fraction structural and functional sperm parameters are grouped and categorised into motility and structural groups. Motility groups include the different motility parameters, kinematics for different speed and progressivity groups (average, rapid, medium, slow and rapid -, medium - and slow-progressive) and hyperactivation as HTF (non-capacitating HTF) and induced (5 mM caffeine, 2 mM procaine and capacitating HTF). Kinematics are further categorized into velocity (VCL, VAP and VSL), linear (LIN and STR) and vigour (WOB, DANCE, BCF and ALH) groups and the structural group into the percentage intact MMP, vitality, immature and mature spermatozoa. Group (II) of the semen parameter groups had the best predictive value towards the HM sperm fractions structural and functional parameters, with semen viscosity contributing the most that model. Significant values indicated in bold as *P<0.05 and **P<0.01. ALH; Amplitude of lateral head displacement, Avg; Average, BCF; Beat cross frequency, Conc; Concentration, Cytopl droplets; Cytoplasmic droplets, DI; Deformity index, HA; Hyperactivation, Head def; Head defect, HM; High motile fraction, HTF; Human tubal fluid, IM; Immature, LIN; Linearity, LM; Low motile fraction, MAI; Multiple anomalies index, Mprog; Medium progressive, Midpiece def; Midpiece defect, MMP; Intact mitochondrial membrane potential, MP; Mucous penetration, Nprog; Non-progressive motility, Norm. acro; Normal acrosome, Norm. morph; Normal morphology, PM; Progressive motility, Prog; Progressive, Rprog; Rapid progressive, ROS; Reactive oxygen species, Sprog; Slow progressive, STR; Straightness, Tail def; Tail defect, TM; Total motility, TZI; Teratozoospermic index, VAP; Average path velocity, VCL; Curvilinear velocity, Visc; Viscosity, Vol; Volume, VSL; Straight-line velocity, WOB; Wobble, R2; R-squared, and b*; Beta coefficients.

Compared to semen groups I and IV (A) and (B), semen group II (total motility, semen viscosity, MPT and progressive motility) had majority R-squared values range above 0.50 for both structural and motility parameters of the HM fraction. However, significance was only seen for percentage rapid swimming spermatozoa, velocity and vigour kinematic parameters of average rapid, medium, and rapid progressive speeds, induced hyperactivation and immature spermatozoa. Consequently, the semen total motility, viscosity, MPT and progressive motility could therefore account for more than 50% of the variation seen in the above-mentioned parameters together. In terms of beta coefficients, semen viscosity had the most impact (positive) on the previously mentioned HM fraction parameters, whereas total motility had the most impact on immature spermatozoa in the HM fraction.

In groups IV (A) and IV (B), R-squared values for morphology parameters ranged above 0.5 for induced hyperactivation and intact MMP; however, significance was only seen for induced hyperactivation. Together, semen morphology parameters can therefore account for more than 50% of the variability seen in induced hyperactivation results of the HM fraction. Beta coefficients of the sperm head defects and normal acrosome in group IV (A), both appeared to have the most impact on induced hyperactivation, whereas in group IV (B), TZI and cytoplasmic droplets had the most impact.

Discussion

The present study evaluated an extensive set of functional and structural parameters of two sperm motility fractions (HM and LM) generated via double DGC of healthy donor semen samples. By collectively comparing a large number [96] of diverse characteristics between motility fractions, and using a new approach of grouping variables to correlate fraction's results to the standard semen analysis, the study provides possible insights into a select group of semen characteristics that could improve its predictive value on the quality of HM sperm subpopulations. Our results indicate significantly enhanced functional and structural characteristics in HM fractions compared to LM fractions. Grouping of various semen characteristics further revealed which of these characteristics relate to a specific fraction (HM or LM), as well as to a specific group of functional or structural variables in that fraction.

Greater values of viable, mature, motile, and morphologically normal spermatozoa have been isolated in HM fractions when compared to neat seam samples (4). Furthermore, several studies have compared the functionality of human sperm subpopulations but were limited in the number of functional and structural parameters investigated. Nonetheless, the latter studies reported higher motility and kinematic parameters in HM fractions compared to LM fractions for both human and bull semen, thereby agreeing with our motility and kinematic parameter findings (8-10). Although average total motility of the HM fraction falls below 90%, it is of note that donor semen was selected to have a non-biased reflection on the functionality of sperm subpopulations in various semen qualities (26). Despite having a large variation in values (range: 40.0-85.5%), total motility in the HM fraction remained than the LM fraction motility range (range: 1.1-3.5.4%). Additionally, we found significantly higher percentages of viable, mature spermatozoa with intact MMP, normal chromatin integrity and ARIC within the HM fraction compared to the LM fraction, which correspond to reports of sperm subpopulations with increased MMP containing more morphologically normal and motile sperm which respond better to induced AR (27-29).

The LM fraction presented with significantly higher levels of immature spermatozoa with positive ROS and abnormal chromatin integrity; all parameters thought to be key etiological causes in idiopathic male infertility (8, 30). Also, previous studies observed lower percentages of mature, motile, morphologically normal spermatozoa with intact chromatin integrity as well as more DNA damage and ROS in LM fractions compared to HM fractions of both donor and patient samples (8). High ROS levels, as documented in semen samples of infertile men, and are known to impair sperm viability and mitochondrial respiration, as well as increase DNA damage and lipid peroxidation (8, 30, 31). The resulting loss of sperm motility and membrane integrity, consequently impair the fusion with the oocyte (8, 30). Considering LM fractions consisted of significantly higher numbers of immature and ROS positive spermatozoa, it is likely such factors contributed to decreased motility, viability, MMP intactness and normal chromatin integrity that our study observed within this fraction. Separation and removal of sperm fractions with elevated levels of immature, ROS positive spermatozoa should thus improve sperm motility and help maintain membrane and chromatin integrity (30).

Higher concentrations of essential proteins involved in sperm functionality and spermatogenesis have been reported in HM fractions, whereas LM fractions consistently presented with alterations and gene expression profiles significantly associated with male infertility (28, 29, 32). These observations were also found to closely coincide with those seen in normospermic samples compared to asthenozoospermic samples (33). Considering the essential function of a selection of these proteins in permitting spermatozoa to undergo capacitation, it is conceivable that we observed significantly higher percentages of induced AR and hyperactivation within the HM fraction compared to the LM fraction (29). We admit that the hyperactivation values presented may be underestimated, as Mortimer et al. (34) have shown that by using VCL and the D-fractal, more accurate hyperactivation percentages can be obtained. However, using the flush technique in a Leja chamber seems to induce maximum hyperactivation of sperm in a more reasonable or accelerated time period to be employed in the clinical laboratory as compared to the traditional swim up technique (23). The LM fraction notably presented with similar structural and functional complications to what have been found in sub-fertile semen samples in terms of levels of immature spermatozoa, ROS, motility parameters hyperactivation and DNA fragmentation (30, 31). Additionally, normozoospermic samples tend to be more homogeneous compared to heterogeneous asthenozoospermic and sub-fertile samples, which closely corresponds to our observations that the HM fraction displayed more homogeneity in sperm functional and structural parameters compared to the LM fraction (32).

According to WHO guidelines (16), clinical human semen analysis is considered an important initial test in evaluating the quality of semen samples and is partly based on the assessment of sperm viability, morphology, concentration and motility (35). Sperm motility is frequently utilized for assessment of semen quality due to its general positive correlation with sperm MMP, concentration, viability and fertilising capacity, while progressive motility is suggested as the most important motility percentage to evaluate in ART programs (2, 3, 36). Nevertheless, it is suggested that more detailed analyses based on distinct sperm subpopulations may disclose other motility parameters and patterns, apart from progressive motility, as good predictors of male fertility (3, 37).

It is clear from our results that grouping of semen total motility, progressive motility, viscosity and MPT presents with higher predictive value towards HM fraction motility and structural grouped parameters in comparison to grouped semen morphology parameters. Interestingly, from this group-semen viscosity had the greatest predictive power towards the HM fraction's grouped motility parameters. Normal semen viscosity plays a critical role in sperm function by facilitating the entry of spermatozoa into cervical mucus, maintaining sperm swimming speed after MPT and preserving chromatin integrity of spermatozoa (38). However, whether the MPT could be a function of the viscosity of the semen, or alternatively a function of the spermatozoa remains difficult to explain as we used the centipoise Leja viscosity test and had unclear results from the correlations (19).

The intrinsic value of combining specific semen parameters into four groups, rather than considering individual semen parameters when assessing relationships with each fraction's sperm parameters should be highlighted. For example, on its own the percentage total motility of the standard semen analysis only had a positive correlation with the motility group of the HM fraction-more specifically the hyper activation subgroup. Interestingly, progressive motility from the basic semen analysis had a positive correlation with the LM fraction's rapid vigour kinematic parameters, but no correlation with the HM fraction. Furthermore; the various semen morphology abnormalities presented with numerous correlations to the LM fraction's lower motility and kinematic parameters, immature spermatozoa, positive ROS and reduced MMP. The strong correlations we found between the semen morphology abnormalities and the LM fraction, in addition to the heterogeneous trend of characteristics within this fraction are likely an attribute of the varying degrees of altered proteins which affect spermatogenesis and ultimately sperm structure and function (28, 29, 32).

Considering our results mentioned above as well as the strong correlations found between the percentage of diverse subpopulations and sperm quality and fertility, the predictive value of basic semen parameters should be re-evaluated (1, 2). For instance, if a high concentration of spermatozoa typical of the LM fraction exists in a semen sample, this can result in the standard semen analysis reflecting the quality of LM fraction, despite the presence of a significantly improved HM fraction that can be separated. Ultimately, such a scenario will result in a skewed semen analysis and subsequent fertility diagnosis. In contrast, if a semen sample contains a high concentration of HM-type spermatozoa, the semen morphology analysis will probably not bear much significance to the functional capabilities of the major sperm population. Similarly, Agarwal and colleagues showed that nine semen characteristics can be grouped and reduced to two scores (semen quality and relative semen quality scores) by principal component analysis, thereby providing a reliable alternative for the prediction of ART outcome in couples with male-factor or idiopathic infertility (39). It is important to note that despite isolating good quality spermatozoa for ART purposes, swim-up protocols result in recovery of motile spermatozoa, whereas DGC separation does not necessarily depend on motile spermatozoa and thus may be utilized for asthenozoospermic samples (40). These findings thereby substantiate the significant role that sperm subpopulations play in factors contributing to male infertility, indicating that even though a semen parameter may fall below the reference value as recommend by WHO (16), possibility of normal fertility should not be excluded as indicated by the different results of the both fractions' functional and structural parameters. As such, the evaluation of subpopulations present in an ejaculate can further assist in the selection of the most suitable treatment or management course to address fertility issues in individual patients or couples.

Conclusion

Our study confirms that spermatozoa of the HM fraction have enhanced functional and structural sperm parameters and display a more homogenous pattern in results amongst individual samples, thereby closely mimicking the functionality and quality of a potentially fertile semen samples. In contrast, separated LM fractions, marked by significantly lower sperm functionality, display more heterogeneous patterns amongst individual sample results and closely mimic functionality and quality of a sub-fertile semen samples. We therefore propose that neat semen samples be separated into sperm subpopulations for both clinical and research purposes. Quantification of functional and structural sperm characteristics for individual fractions may provide more accurate reflections of sperm and semen quality and improve the prediction and diagnosis of the fertilization potential of the whole ejaculate, especially in sub-fertile semen cases. Such sperm fractions can further be utilized as a potential research model of sperm physiology for investigating "fertile" and "sub-fertile" samples. In future, such a model should be utilized to investigate how spermatozoa from different subpopulations respond to various treatments and subsequently could provide insights on how to improve the functionality of sub-fertile semen samples to approximate that of fertile semen samples. Finally, when focusing on an individual semen trait as a possible predictor for male fertility, such as progressive motility, this may result in an over- or underestimated prediction. In contrast, using a combined group of related semen traits may elude more information into a specific group and even sub-group of the functional and structural variables of either sperm motility fraction relating to fertility. The grouped combinations of traits may compensate for an individual trait of poor quality, thereby producing more accurate estimations of overall functional quality of the spermatozoa.

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Authors' Contributions

L.M., S.K., G.v.d.H.; Conceptualization, design, methodology, and software. S.K., L.M.; Validation, visualization, project administration, and funding acquisition. S.K.; Formal analysis, investigation, data acquisition, interpretation, data curation, and writing-original draft preparation. L.M., G.v.d.H.; Resources, writing-review and editing, and supervision. All authors read and approved the final manuscript.

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