

Basic semen parameters assisted by Computer-Aided sperm analysis (CASA) and their correlations with advanced semen parameters in normozoospermic men with different abstinence periods

by

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Declaration

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Abstract

Introduction: Affordable basic semen analysis remains a fundamental procedure to be performed routinely during the diagnosis of male infertility. Advanced semen analyses, provide valuable clinical insights in treatment related decision-making, but these are highly expensive and lack universal standardization. The World Health Organization (WHO) guidelines for semen analysis have been adopted by most human andrology and fertility laboratories around the world for more than thirty years. According to the most recent prescribed guidelines of the WHO, subjects must remain abstinent for a minimum period of two days, but not longer than seven days before collecting a sample for a standard semen analysis. Several studies have sought to determine the optimal period for ejaculatory abstinence. However, the results are often found to be contradictory.

The aims of this study are two-fold:

Aim I: To investigate the effect of short (4 hours) and long (4 days) abstinence periods on sperm quality based on functional and biochemical parameters in a population of normozoospermic men, in addition to the prediction of various basic and advanced semen parameters of the second (4 hours) ejaculate from a set of basic parameters obtained from the first (4 days) ejaculate.

Aim II: Establishing a correlation between basic semen parameters assisted by Computer-aided sperm analysis (CASA) and a set of advanced semen analysis tests. To determine cut-off values for advanced semen parameters from various basic parameters based on WHO defined reference values.

Methods: Semen samples were collected from one hundred potentially fertile, normozoospermic men (20 to 30 years) who abstained for a period of exactly 4 days and 4 hours prior to collection of the first and second ejaculates respectively. Semen samples were analysed according to the WHO guidelines. Sperm concentration, total sperm count (T.S.C.), total and progressive motility and kinematic/velocity parameters were analysed by CASA. Sperm viability was performed by dye exclusion and morphology via SpermBlue™ staining techniques using Computer Aided Sperm Morphology Analysis (CASMA). Sperm acrosome status was evaluated by fluorescence microscopy. Sperm DNA fragmentation and intracellular superoxide ($O_2^{\cdot-}$) levels were assessed by flow cytometry. Seminal antioxidant status [superoxide dismutase (SOD), catalase (CAT), thiobarbituric acid reactive substances (TBARS)] were measured by means of spectrophotometry.

Statistical comparisons between short and long abstinence periods were performed using paired Student's t-tests on GraphPad Prism™ software, while the prediction of various basic and advanced semen parameters of the second ejaculate from a set of basic semen parameters of the first ejaculate was performed using linear regression models. Correlations were performed using Spearman rank

correlation coefficients, while receiver operating characteristic (ROC) curves were used to determine cut-off values. Statistical significance was set at $p < 0.05$.

Results I: A significant increase in total and progressive motility as well as in the velocity parameters were observed after short (4 hours) abstinence compared to long (4 days) abstinence periods. DNA fragmentation and intracellular $O_2^{\cdot-}$ levels were not significantly different between short and long abstinence periods. Despite the observed decrease in semen volume, sperm concentration and T.S.C. after the short abstinence period, all mean values of the conventional semen parameters still remained above the lower reference limits as recommended by the WHO 5th edition. We were also able to make predictions of various basic (semen volume, sperm concentration, total motility, progressive motility, viability and normal morphology) and advanced (DNA fragmentation, seminal plasma CAT activity and TBARS) parameters of the second ejaculate from a set of basic semen parameters obtained from the first ejaculate with relative certainty.

Results II: The proportions of total and progressively motile as well as rapid spermatozoa were positively correlated with CAT activity ($p < 0.05$). A significant negative correlation was observed between VCL, VSL, VAP and both intracellular $O_2^{\cdot-}$ and TBARS levels. ALH was significantly and negatively correlated with intracellular $O_2^{\cdot-}$ levels and DNA fragmentation, while its correlation with SOD activity was positive ($p < 0.05$). A negative correlation was also found between the percentage of viable spermatozoa and both $O_2^{\cdot-}$ levels and DNA fragmentation, whereas the percentage of normal morphology was negatively correlated with $O_2^{\cdot-}$ levels and positively with CAT activity ($p < 0.05$). The optimal intracellular $O_2^{\cdot-}$ cut-off value to differentiate between asthenozoospermic and normozoospermic men was calculated to be 227 median DHE fluorescence intensity [MFI] ($p < 0.01$). At this cut-off value, the test was 80% sensitive and 86% specific. Sperm viability was associated with a seminal plasma TBARS cut-off value of 9.86 $\mu\text{mol/L}$ ($p = 0.02$) with sensitivity and specificity of 81% and 80% respectively.

Conclusion: Our data challenges the generally accepted guidelines regarding the prescribed prolonged abstinence periods since the results show that 4 hours of sexual abstinence yielded significantly better samples from a sperm functional point of view. The results obtained from this study further support the validity of some CASA parameters as sensitive indicators of changes in sperm oxidative status and DNA integrity. This study also enabled defining the cut-off values and prediction of certain advanced variables from the basic semen analysis.

Opsomming

Inleiding: Bekostigbare basiese semenanalise bly ‘n fundamentele prosedure wat roetinegewys uitgevoer word tydens die diagnose van manlike onvrugbaarheid. Gevorderde semenanalises bied waardevolle kliniese insigte in behandelingsverwante besluitneming, maar dit is baie duur en is nie universeel gestandaardiseer nie. Die Wêreldgesondheidsorganisasie (WGO) se riglyne vir die analisering van semen word wêreldwyd al vir meer as dertig jaar deur die meeste menslike andrologie- en vrugbaarheidslaboratoriums aangeneem. Volgens die mees onlangse voorgeskrewe riglyne van die WGO, moet mans hulself onthou van ejakulasie vir ‘n minimum tydperk van twee dae, maar nie meer as sewe dae lank voor die versameling van ‘n monster vir ‘n standaard semenanalise nie. Verskeie studies het probeer om die optimale tydperk vir ejakulatoriese onthouding te bepaal, maar die bevindinge was dikwels teenstrydig.

Die doelstellings van hierdie studie is tweevoudig:

Doelwit I: Om die effek van kort (4 uur) en lang (4 dae) onthoudingsperiodes op spermkwaliteit te ondersoek, gebaseer op funksionele en biochemiese parameters in ‘n populasie van normozoospermiese mans; en benewens daartoe om verskillende basiese en gevorderde semenparameters van die tweede (4 uur) ejakulaat te voorspel gebaseer op ‘n stel basiese parameters verkry vanaf die eerste (4 dae) ejakulasie.

Doelwit II: Die vasstelling van korrelasies tussen basiese semenparameters, bygestaan deur rekenaargesteuende spermanalise (CASA), en ‘n stel gevorderde semenanalise toetse. Om afsnyppunte vir gevorderde semenparameters te bepaal uit verskeie basiese parameters gebaseer op WGO gedefinieerde verwysingswaardes.

Metodes: Semenmonsters is versamel van een honderd potensieel vrugbare, normozoospermiese mans (20 tot 30 jaar) wat vir ‘n tydperk van presies 4 dae en 4 ure voor die eerste en tweede ejakulasie onderskeidelik onthou het. Semenmonsters is volgens die WGO-riglyne ontleed. Spermkonsentrasie, totale spermtelling (T.S.C.), totale en progressiewe motiliteit en kinematiese / snelheid parameters is deur CASA geanaliseer. Spermlewensvatbaarheid is bepaal deur middel van kleurstofuitsluiting en morfologie met behulp van SpermBlue™ kleuringstegnieke en die gebruikmaking van rekenaargesteuende spermmorfologie-analise (CASMA). Akrosoom status is bepaal deur fluoresensie mikroskopie. DNA-fragmentasie en intrasellulêre superoksied ($O_2^{\cdot-}$) vlakke is gemeet met behulp van vloeisitometrie. Seminale antioksidante status [superoksied dismutase (SOD), katalase (CAT), tiobarbituriensuur reaktiewe substansie (TBARS)] is geassesseer met behulp van spektrofotometrie.

Statistiese vergelykings tussen kort en lang onthoudingsperiodes is uitgevoer deur gebruik te maak van gepaarde Student t-toetse op GraphPad Prism™ -programmatuur, terwyl die voorspelling van

verskeie basiese en gevorderde semenparameters van die tweede ejakulasie vanaf 'n stel basiese semenparameters van die eerste ejakulaat uitgevoer is deur gebruik te maak van lineêre regressiemodelle. Korrelasies is uitgevoer met behulp van die Spearman-rangkorrelasie koëffisiënt, terwyl operasionele karakteristieke krommes van ontvanger (ROC) gebruik is om afsny punte te bepaal. Statistiese betekenisvolheid is vasgestel op $p < 0.05$.

Resultate I: 'n Beduidende toename in totale en progressiewe motiliteit sowel as in die snelheidsparameters is waargeneem na kort (4 uur) onthouding in vergelyking met lang (4 dae) onthoudingsperiodes. DNA fragmentasie en intrasellulêre O_2^{\bullet} vlakke was nie beduidend verskillend tussen kort en lang onthoudingsperiodes nie. Ten spyte van die waargeneemde afname in semen volume, sperm konsentrasie en T.S.C. na die kort onthoudingsperiode, bly alle gemiddelde waardes van die konvensionele semenparameters steeds bo die laer verwysingslimiete soos aanbeveel deur die WGO 5^{de} uitgawe. Ons was ook in staat om voorspellings met relatiewe sekerheid te maak van verskillende basiese (semen volume, spermkonsentrasie, totale motiliteit, progressiewe motiliteit, lewensvatbaarheid en normale morfologie) en gevorderde (DNA-fragmentasie, plasma-CAT-aktiwiteit en TBARS) parameters van die tweede ejakulasie vanaf 'n stel basiese semen parameters verkry vanaf die eerste ejakulasie.

Resultate II: Die verhoudings van totale en progressief motiele sowel as vinnige spermatozoa was positief gekorreleer met CAT aktiwiteit ($p < 0.05$). 'n Beduidende negatiewe korrelasie is waargeneem tussen VCL, VSL, VAP en beide intrasellulêre O_2^{\bullet} en TBARS-vlakke. ALH was beduidend en negatief gekorreleer met intrasellulêre O_2^{\bullet} vlakke en DNA-fragmentering, terwyl die korrelasie met SOD-aktiwiteit positief was ($p < 0.05$). 'n Negatiewe korrelasie is ook gevind tussen die persentasie lewensvatbare spermatozoa en beide O_2^{\bullet} en DNA-fragmentering, terwyl die persentasie normale morfologie negatief gekorreleer is met O_2^{\bullet} vlakke en positief met CAT-aktiwiteit ($p < 0.05$). Die optimale intrasellulêre O_2^{\bullet} afsnywaarde om te onderskei tussen astenozoospermiese en normozoospermiese mans is bereken as 227 mediaan DHE fluoresensie-intensiteit [MFI] ($p < 0.01$). By hierdie afsnywaarde was die toets 80% sensitief en 86% spesifiek. Spermlewensvatbaarheid is geassosieer met 'n seminale plasma TBARS-afsnijwaarde van 9.86 $\mu\text{mol} / \text{L}$ ($p = 0.02$) met 'n sensitiwiteit en spesifisiteit van onderskeidelik 81% en 80%.

Gevolgtrekking: Ons data daag die algemeen aanvaarde riglyne uit rakende die voorgeskrewe verlengde onthoudingsperiodes aangesien die uitslae aantoon dat 4 uur seksuele onthouding aansienlik beter semenmonsters oplewer soos beskou uit 'n spermfunksionele oogpunt. Die resultate soos verkry uit hierdie studie, ondersteun verder die geldigheid van sommige CASA parameters as sensitiewe aanwysers van veranderinge in sperm oksidatiewe status en DNA integriteit. Hierdie studie

het ook die definiëring van afsnywaardes en voorspelling van sekere gevorderde veranderlikes vanaf die basiese semenanalise bepaal.

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Table of Contents

Declaration	ii
Abstract.....	ii
Opsomming	iv
Acknowledgements	vii
Table of Contents	viii
List of Tables	xiii
List of Figures.....	xiv
Abbreviations	xviii
Chapter 1: Introduction	1
1.1 Background.....	1
1.2 Motivation of the study.....	7
1.2.1 Aim I: Effect of abstinence period on semen quality.	7
1.2.2 Aim II: Relationship between advanced and basic semen parameters	8
1.3 Outline of the study.....	9
1.4 Aims and objectives.....	10
Chapter 2: Literature Review	11
2.1 Sperm development/spermatogenesis	11
2.1.1 Mitosis:	12
2.1.2 Meiosis:.....	14
2.1.3 Spermiogenesis:	15
2.1.4 Epididymal maturation	16

2.1.5	Storage of spermatozoa in the epididymis	16
2.2	Structure of human spermatozoa	17
2.2.1	The head.....	18
2.2.2	The neck.....	19
2.2.3	The midpiece	20
2.2.4	The tail	21
2.2.5	The axoneme.....	22
2.3	Semen parameters	25
2.3.1	Semen viscosity	25
2.3.2	Semen pH.....	26
2.3.3	Semen volume	27
2.3.4	Sperm Concentration	28
2.3.5	Sperm viability.....	28
2.3.6	Sperm morphology	28
2.3.7	Sperm motility	30
2.3.8	Sperm kinematic movement	32
2.3.9	Sperm hyperactivity.....	35
2.3.10	Acrosome reaction	37
2.3.11	Sperm DNA fragmentation.....	39
2.3.12	Reactive oxygen species (ROS).....	42
2.3.12.1	Formation of ROS	42
2.3.12.2	Origins of ROS in semen.....	43
2.3.12.3	Physiological roles of ROS.....	45
2.3.12.4	Pathological effects of ROS on spermatozoa	48
2.3.13	Antioxidants.....	50
2.3.13.1	Enzymatic antioxidants.....	50
2.3.13.2	Non-enzymatic antioxidants	52

2.4	Computer-aided sperm analysis (CASA).....	53
2.4.1	Advantages of CASA	55
2.4.1.1	Sperm motility	55
2.4.1.2	Sperm kinematics	55
2.4.1.3	Sperm hyperactivation	56
2.4.1.4	Sperm morphology	56
2.4.1.5	Sperm morphometry	57
2.4.2	Clinical relevance of CASA measurements	58
2.4.3	Limitations of CASA	59
2.4.4	Future of CASA	60
2.5	Factors affecting sperm quality.....	61
2.5.1	Age.....	61
2.5.2	Smoking.....	62
2.5.3	Alcohol consumption.....	62
2.5.4	Obesity.....	63
2.5.5	Recreational drugs	63
2.5.6	Ejaculatory abstinence	64
Chapter 3: Materials and Methods		67
3.1	Study volunteers.....	67
3.2	Basic Semen analysis.....	69
3.2.1	Semen sample collection	69
3.2.2	Initial macroscopic examination.....	69
3.2.3	Volume	69
3.2.4	The pH	70
3.2.5	Viscosity	70
3.2.6	Sperm concentration and motility.....	70
3.2.7	Sperm morphology	74

3.2.8	Sperm viability.....	75
3.3	Advanced semen analysis	77
3.3.1	Acrosome reaction	77
3.3.2	DNA fragmentation	78
3.3.3	Intracellular Superoxide ($O_2^{\cdot-}$)	80
3.3.3.1	Flow cytometry analysis.....	81
3.3.4	Seminal plasma TBARS and antioxidant assays	84
3.3.4.1	Sample preparation	84
3.3.4.2	TBARS assay.....	84
3.3.4.3	Catalase (CAT) activity assay	85
3.3.4.4	Superoxide dismutase (SOD) activity assay	85
3.4	Statistical analysis.....	87
3.4.1	Aim I: Effect of abstinence period on semen quality.	87
3.4.2	Aim II: Relationship between advanced and basic semen parameters	89
Chapter 4: Results.....		90
4.1	Aim I: Effect of abstinence period on semen and sperm quality.	90
4.1.1	Influence of abstinence period on basic semen parameters.....	90
4.1.2	Influence of abstinence period on advanced semen parameters	102
4.2	Aim II: Relationship between basic and advanced semen parameters.	105
4.2.1	Correlation between basic and advanced semen parameters	105
4.2.2	Establishing cut-off values for the advanced semen parameters from basic semen parameters.....	121
Chapter 5: Discussion.....		130
5.1	Aim I: Effect of abstinence period on semen quality.	130
5.1.1	Ejaculatory abstinence and basic semen parameters	130
5.1.1.1	Semen pH	130

5.1.1.2	Semen Volume	132
5.1.1.3	Sperm Concentration	134
5.1.1.4	Sperm Viability.....	136
5.1.1.5	Sperm Morphology.....	136
5.1.1.6	Sperm Motility.....	138
5.1.2	Ejaculatory abstinence and advanced semen parameters	143
5.1.2.1	Acrosome Reaction.....	144
5.1.2.2	DNA Fragmentation	145
5.1.2.3	ROS generation and oxidative stress markers	146
5.2	Aim II: Relationship between basic and advanced semen parameters	149
5.2.1	Intracellular O ₂ ^{-•} levels.....	149
5.2.2	Seminal plasma lipid peroxidation	152
5.2.3	Seminal plasma antioxidant activity.....	155
5.2.4	Acrosome Reaction.....	158
5.2.5	DNA Fragmentation	159
Chapter 6: Conclusion		162
6.1	Aim I: Effect of abstinence period on semen quality	162
6.2	Aim II: Relationship between basic and advanced semen parameters.	163
6.3	Limitations/recommendations of the study.....	164
6.4	Future studies	165
6.5	Research Outputs	169
References		171
Appendix		224

List of Tables

Table 3. 1: Camera settings for the SCA® system	71
Table 3. 2: SCA® concentration, motility and kinematic parameters	73
Table 4. 1: Semen characteristics in short vs. long abstinence	91
Table 4. 2: sperm motility characteristics in short vs. long abstinence	94
Table 4. 3: Sperm velocity parameters in short vs. long abstinence	96
Table 4. 4: Sperm kinematic parameters in short vs. long abstinence	99
Table 4. 5: Advanced semen parameters in short vs. long abstinence.....	102
Table 4. 6: Correlation analysis between basic and advanced parameters	106
Table 4. 7: Correlation between sperm motility/velocity and advanced parameters.....	108
Table 4. 8: Correlation analysis between sperm kinematics and advanced parameters.	115
Table A 1. Linear regression summary for the dependent variable: Semen Volume	224
Table A 2. Linear regression summary for the dependent variable: Sperm Concentration...	227
Table A 3. Linear regression summary for the dependent variable: Sperm Viability	230
Table A 4. Linear regression summary for the dependent variable: Normal Morphology ...	233
Table A 5. Linear regression summary for the dependent variable: Progressive Motility	236
Table A 6. Linear regression summary for the dependent variable: Total Motility	239
Table A 7. Linear regression summary for the dependent variable: DNA Fragmentation....	242
Table A 8. Linear regression summary for the dependent variable: Intracellular O ₂ ^{-•}	245
Table A 9. Linear regression summary for the dependent variable: TBARS levels	248
Table A 10. Linear regression summary for the dependent variable: CAT activity	251
Table A 11. Linear regression summary for the dependent variable: SOD activity.....	254

List of Figures

Figure 2. 1: The major events in human spermatogenesis	13
Figure 2. 2: The main cell types that occur in the human seminiferous epithelium	14
Figure 2. 3: Structure of the human sperm.....	20
Figure 2. 4: Structure of the human sperm axoneme.	22
Figure 2. 5: The main kinematic measurements involved in sperm tracking.....	33
Figure 2. 6: CASA-related publications	54
Figure 3. 1: Flow chart showing a simplified experimental protocol	68
Figure 3. 2: SCA® different colour paths for motility rating	72
Figure 3. 3: SCA morphology analysis of spermatozoa stained with SpermBlue.	75
Figure 3. 4: Viability assessment of spermatozoa stained with dye exclusion.	76
Figure 3. 5: Acrosomal status of spermatozoa as seen under florescent microscope	78
Figure 3. 6: Sperm DNA Fragmentation Analysis Using the TUNEL Assay.....	79
Figure 3. 7: Flow cytometry plots (dot plots and histograms)	82
Figure 3. 8: Flow cytometry histogram for the sperm intracellular $O_2^{\cdot-}$	83
Figure 4. 1: Differences in basic parameters between short and long abstinence periods.	92
Figure 4. 2: Star symbol plots comparing six basic semen parameters between short and long periods of abstinence.	93
Figure 4. 3: Differences in motility parameters between short and long abstinence periods	95
Figure 4. 4: Differences in velocity parameters between short and long abstinence periods	97
Figure 4. 5: Star symbol plots comparing five motility parameters between short and long periods of abstinence.	98
Figure 4. 6: Differences in sperm kinematic parameters between short and long abstinence periods.....	100
Figure 4. 7: Star symbol Plots comparing eight kinematic parameters between short and long periods of abstinence.	101
Figure 4. 8: Differences in Advanced Semen Parameters between short and long abstinence periods.....	103
Figure 4. 9: Star symbol Plots comparing five advanced semen parameters between long and short periods of abstinence	104

Figure 4. 10: Correlations of seminal plasma catalase and SOD activity with basic motility parameters.....	109
Figure 4. 11: Correlations of intracellular $O_2^{\cdot-}$ with basic motility parameters.....	110
Figure 4. 12: Correlations of seminal TBARS levels with basic motility parameters.	111
Figure 4. 13: Correlations of sperm acrosome-intact with basic motility parameters.	112
Figure 4. 14: Correlations of sperm DNA fragmentation with basic motility parameters.....	113
Figure 4. 15: Correlations of sperm intracellular $O_2^{\cdot-}$ with kinematic parameters	116
Figure 4. 16: Correlations of seminal plasma TBARS with sperm kinematic parameters.	117
Figure 4. 17: Correlations of seminal plasma catalase and SOD activity with sperm kinematic parameters.....	118
Figure 4. 18: Correlations of sperm acrosome-intact with kinematic parameters.....	119
Figure 4. 19: Correlations of sperm DNA fragmentation with kinematic parameters.	120
Figure 4. 20: Receiver operator characteristic curve showing $O_2^{\cdot-}$ cut-off values and distribution of $O_2^{\cdot-}$ values in samples with normal and below WHO total motility reference values.	121
Figure 4. 21: Receiver operator characteristic curve showing $O_2^{\cdot-}$ cut-off value and the distribution of $O_2^{\cdot-}$ values in samples with normal and below WHO progressive motility reference values.....	122
Figure 4. 22: Receiver operator characteristic curve showing seminal catalase activity cut-off value and distribution of catalase activity values in samples with normal and below WHO progressive motility reference values.....	123
Figure 4. 23: Receiver operator characteristic curve showing TBARS cut-off value and the distribution of TBARS values in samples with normal and below WHO progressive motility reference values..	124
Figure 4. 24: Receiver operator characteristic curve showing seminal SOD activity cut-off value and the distribution of SOD activity values in samples with normal and below WHO progressive motility reference values.	125
Figure 4. 25: Receiver operator characteristic curve showing DNA fragmentation cut-off value and the distribution of DNA fragmentation percentages in samples with normal and below WHO progressive motility reference values..	126
Figure 4. 26: Receiver operator characteristic curve showing TBARS cut-off value and the distribution of TBARS values in samples with normal and below WHO viability reference values	127
Figure 4. 27: Receiver operator characteristic curve showing seminal catalase activity cut-off value and distribution of catalase activity values in samples with normal and below WHO sperm viability reference values.....	128

Figure 4. 28: Receiver operator characteristic (ROC) curve showing seminal SOD activity cut-off value and the distribution of catalase activity values in samples with normal and below WHO sperm viability reference values.	129
Figure A 1: Regression analysis between predicted variable (semen volume of the 2 nd ejaculate) and he residual scores	225
Figure A 2: Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the semen volume of the second ejaculate as a dependent variable.....	226
Figure A 3. Regression analysis between predicted variable (sperm concentration of the 2 nd ejaculate) and the residual scores	228
Figure A 4. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm concentration of the second ejaculate as a dependent variable.....	229
Figure A 5. Regression analysis between predicted variable (sperm viability of the 2 nd ejaculate) and the residual scores	231
Figure A 6. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm viability of the second ejaculate as a dependent variable.....	232
Figure A 7. Regression analysis between predicted variable (sperm normal morphology of the 2 nd ejaculate) and the residual scores.....	234
Figure A 8. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm normal morphology of the second ejaculate as a dependent variable.....	235
Figure A 9. Regression analysis between predicted variable (Progressive Motility of the 2 nd ejaculate) and the residual scores	237
Figure A 10. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm progressive motility of the second ejaculate as a dependent variable.....	238
Figure A 11. Regression analysis between predicted variable (Sperm Motility of the 2 nd ejaculate) and the residual scores.	240
Figure A 12. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm motility of the second ejaculate as a dependent variable.....	241
Figure A 13. Regression analysis between predicted variable (DNA fragmentation of the 2 nd ejaculate) and the residual scores	243
Figure A 14. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm DNA fragmentation of the second ejaculate as a dependent variable.	244

Figure A 15. Regression analysis between predicted (sperm intracellular $O_2^{\cdot-}$ of the 2nd ejaculate) and the residual scores246

Figure A 16. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm $O_2^{\cdot-}$ of the second ejaculate as a dependent variable.....247

Figure A 17. Regression analysis between predicted (seminal plasma TBARS of the 2nd ejaculate) and the residual scores249

Figure A 18. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma TBARS levels of the second ejaculate as a dependent variable.250

Figure A 19. Regression analysis between predicted (seminal plasma CAT activity of the 2nd ejaculate) and the residual scores252

Figure A 20. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma CAT activity of the second ejaculate as a dependent variable.253

Figure A 21. Regression analysis between predicted (seminal plasma SOD activity of the 2nd ejaculate) and the residual scores..255

Figure A 22. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma SOD activity of the second ejaculate as a dependent variable.256

Abbreviations

ART	Assisted Reproductive Technology
ASF	acrosome stabilizing factor
μL	Microliter
μm	Micrometre
ALH	Amplitude of lateral head displacement
BCF	Beat/cross frequency
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine 3', 5' monophosphate
CASA	Computer-aided sperm analysis
CAT	Catalase
CuZn-SOD	Cooper-zinc SOD
DNA	Deoxyribonucleic acid
DRC	Dynein Regulatory Complex
dUTP	Terminal deoxynucleotidyl transferase
EC-SOD	Secretory tetrameric extracellular-SOD
ESHRE	European Society of Human Reproduction and Embryology
FITC	Fluorescein isothiocyanate
g	Gram
G6PD	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidises
H_2O_2	Hydrogen peroxide

H ₂ O	Water molecule
HO ₂	Hydroperoxyl
HAMS	Human albumin serum
HREC	Health Research Ethics Committee
Hz	Hertz
ICSI	Intracytoplasmic Sperm Injection
ISNT	Situ nick translation
IUI	Intrauterine insemination
IVF	In vitro fertilization
L	Litre
LIN	Linearity
MAD	Mean angular displacement
MDA	Malondialdehyde
min	Minute
mL	Millilitre
mM	Millimolar
Mn-SOD	Manganese SOD
N ⁺	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFA	Nordic Association for Andrology
nm	Nanometre
NO [•]	Nitric oxide

NO_2^\bullet	Nitrogen dioxide
O_2	Diatomic oxygen
$\text{O}_2^{\bullet-}$	Superoxide
O_2^{2-}	Peroxide
OH^\bullet	Hydroxyl
OHCl^\bullet	Hypochlorite radical
ONOO^-	Peroxynitrite
PBS	Phosphate Buffered Saline
Pc	Centipoise
PI	Propidium Iodide
PKA	Protein kinase A
PNA	Peanut Agglutinin
PSA	Pisum sativum agglutinin
PUFA	polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
SD	Standard D
SCA®	Sperm Class Analyzer
SCD	Sperm chromatin dispersion test
SCSA	Sperm chromatin structure assay
SOD	Superoxide Dismutase
SPMI	seminal plasma motility inhibitor

STR	Straightness
SURRG	Stellenbosch University Reproductive Research Group
TAC	total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
TdT	Terminal Deoxynucleotidyl Transferase
TUNEL	Deoxynucleotidyl transferase dUTP nick end labeling
VAP	Average-path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WHO	World Health Organization
WOB	Wobble
WST	Water-soluble tetrazolium salt

Chapter 1: Introduction

1.1 Background

Infertility is delineated by the International Committee for Monitoring Assisted Reproductive Technology, World Health Organization (WHO), as “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse”. Globally, an estimated 60–80 million couples of reproductive age currently suffer from infertility (WHO, 2004). The prevalence of infertility diverges widely across regions and is estimated to affect an average of 8 to 12 % of couples around the world (Kumar and Singh, 2015). Male factor infertility has been shown to be the sole contributor in approximately 20 % of all infertility cases and is partially implicated in another 30–40 % (Sharlip et al., 2002). Usually, when the attributable causes of female infertility have been eliminated and/or semen analysis results fail to meet the WHO criteria (WHO, 2010), male infertility is taken into consideration as the likely etiological factor.

In recent years, the overall incidence of male infertility has increased dramatically, reflecting a progressive decline in semen quality and a concurrent increase in abnormalities of the male reproductive tract (Kumar and Singh, 2015). An emerging concern has been raised about the global time-related deterioration in semen quality. For instance, in a meta-analysis, which was carried out between 1938 and 1991 and included 61 studies from multiple nations, a substantial reduction in the mean sperm concentration, from $113 \times 10^6/\text{mL}$ to $66 \times 10^6/\text{mL}$, was reported among men with no prior history of infertility. This indicates that sperm concentration had declined worldwide by an average of 50 % during the last 50 years of the twentieth century (Carlsen et al., 1992). These findings attracted a considerable attention and have been supported by several recent studies that consistently revealed a global downward trend in semen quality (Borges et al., 2015, Huang et al., 2017, Sengupta et al., 2017).

A considerable amount of variability has also been shown to exist in various semen characteristics within and among individuals (Alvarez et al., 2003). These variations have been largely attributed to

several modifiable intrinsic and extrinsic factors. These factors include the length of sexual abstinence, ejaculation frequency and method of collection. Other factors that have the potential to influence semen quality are general health and lifestyle, infection, dysfunction of male sex glands, urogenital surgery as well as therapeutic and environmental exposures (Bahadur et al., 2016a; Du Plessis et al., 2014; Gosálvez et al., 2011; Valsa et al., 2013).

The World Health Organization (WHO) manuals for examining and processing human semen (WHO, 2010) provide a practical guide for standardizing semen analysis. These manuals have been periodically published and actively revised since its first edition in 1980. The WHO criteria for semen analysis have been adopted by most human andrology and fertility laboratories around the world for more than thirty years. According to the most recent prescribed guidelines of the WHO, subjects must remain abstinent for a minimum period of two days, but not longer than seven days before collecting a sample for a standard semen analysis (WHO, 2010). More constricted abstinence intervals of three to four days have also been suggested by the Nordic Association for Andrology (NAFA) and the European Society of Human Reproduction and Embryology [ESHRE] (Kvist and Björndahl, 2002). The basis for these recommendations is nevertheless not supported by sufficient scientific evidence and requires further clarification.

In light of the differing ejaculatory abstinence periods suggested by various regulatory bodies, a growing concern has resulted over what the precise period of ejaculatory abstinence ought to be for an optimal semen sample. This has prompted several studies to examine the influence of abstinence periods on various semen quality parameters; however, the results are not conclusive. Interestingly, some studies have even challenged the recommended guidelines in favour of extremely shorter periods (i.e. > 1 hour to 4 hours) due to their advantageous effects on semen characteristics (Bahadur et al., 2016a; Gosálvez et al., 2011; Mayorga-Torres et al., 2016; Valsa et al., 2013).

During the last half century, several studies have sought to determine the optimal time frame for ejaculatory abstinence, however the results are often found to be contradictory. In general, these studies assessed a wide range of abstinence period cut-offs (<1–18 days). Prolonged periods of sexual

abstinence have generally been reported to increase semen volume, total sperm count (T.S.C.) and sperm concentration (Agarwal et al., 2016; Marshburn et al., 2014; Mayorga-Torres et al., 2015; Sunanda et al., 2014). However, the overall quality of spermatozoa has shown to be influenced by the efficiency of epididymal storage and the transit rate of spermatozoa, which is apparently dependent on the frequency of ejaculation (Johnson and Varner, 1988; Turner, 2008).

Progressive motility (Bahadur et al., 2016a) as well as the percentage of motile spermatozoa (Agarwal et al., 2016; Choavaratana et al., 2014; Valsa et al., 2013) were found to decrease substantially with increased abstinence period, while no significant differences were observed in other studies (Jurema et al., 2005; Mayorga-Torres et al., 2015; Sánchez-Martín et al., 2013). In addition, only a few studies are available on the impact of abstinence time on advanced sperm functional parameters such as intracellular Reactive Oxygen Species (ROS) production and DNA integrity, while their findings are apparently inconsistent. Shortening the abstinence time resulted in a significant decrease in sperm DNA fragmentation (Agarwal et al., 2016; Sánchez-Martín et al., 2013; Sukprasert et al., 2013) and ROS levels (Mayorga-Torres et al., 2016), whereas other studies reported no significant differences (Mayorga-Torres et al., 2015; De Jonge et al., 2004; Desai et al., 2010).

Chronobiological studies showed that various changes occur in the body every 4 hours (Valsa et al., 2013). Despite this, no comprehensive study has been undertaken to investigate the effect of a short abstinence period lasting 4 hours on conventional semen parameters, in addition to various existing sperm functional parameters such as acrosome reaction, sperm ROS and DNA fragmentation, and seminal plasma antioxidant capacity. Accordingly, the typical time after which semen samples should be collected for standardized analysis remains unclear and needs to be further investigated.

In addition to a detailed medical history and a thorough physical examination, conventional semen analysis remains a fundamental procedure performed on routine basis during the diagnosis of male infertility (Hamada et al., 2012). Being cost-effective and not technically demanding, semen analysis is largely used as a preliminary diagnostic tool for the evaluation of male infertility (Sikka and Hellstrom, 2016; Vasan, 2011). The analysis provides essential information about the basic

characteristics of the semen which include the ejaculate volume, sperm concentration, motility as well as viability and morphology. However, conventional semen analysis, if performed manually, is criticized for being subjective, time consuming and prone to inter- and intra- laboratory variations related especially to the identification of motile sperm subpopulations (Cooper and Yeung, 2006) and morphology assessment (Daoud et al., 2016; Rivera-Montes et al., 2013). The potential counting and interpretation errors associated with the subjective visual assessment of the traditional semen analysis have highlighted the absolute necessity for computerized systems designed to automate the analysis. In contrast to the manual analysis, computer-aided sperm analysis (CASA) system, if used proficiently under identical settings, is undeniably a powerful approach for the objective assessment of spermatozoa. CASA is an automated system with hardware and software packages designed to visualize and digitalize series of sequential images of spermatozoa (Lu et al., 2014; Talarczyk-Desole et al., 2017). The obtained data is processed and analysed to provide the users with quantitative information about different aspects of sperm quality (Amann and Katz, 2004). On the basis of better computer technology and image resolution, the commercially available CASA systems not only measure the kinematic parameters more accurately, but also in closer agreement with each other (Mortimer et al., 2015).

The WHO laboratory manuals for the examination and processing of human semen provide a primary reference guideline for standardizing semen analysis. These manuals have been periodically published and actively developed since its first edition in 1980 (WHO, 1980, 1987, 1992, 1999, 2010). Most of the traditional and automated semen analysis methods have aligned their measurements according to these criteria (Lu et al., 2014; Mortimer et al., 2015). The inclusion of normal reference values of semen parameters in the WHO manuals has been of enormous significance in establishing some consistency regarding the basic characteristics of normal ejaculate (Björndahl, 2011).

However, semen parameter values do not necessarily reflect the functional integrity of spermatozoa, and studies have revealed a significant overlap in the semen characteristics between fertile and infertile men (Hamada et al., 2012; Lewis, 2007). Consequently, a large proportion of men with

normal semen analysis results are often diagnosed as having unexplained infertility as the underlying pathophysiology of sperm functional deficiencies remains largely unknown (Khodair and Omran, 2013; Sikka and Hellstrom, 2016).

The recent enormous progress towards understanding the biochemical and molecular mechanisms regulating human sperm function has driven the development of a variety of assays for proper evaluation of the functional quality of spermatozoa (Franken and Oehninger; 2012; Sikka and Hellstrom, 2016; Van der Horst and Du Plessis, 2017). These assays provide valuable clinical insights into multiple aspects of sperm function, including DNA integrity as well as oxidative stress and membrane lipid peroxidation. The obtained information could be of highest value to assist the clinician in treatment-related decision-making (Lamb, 2010, Oehninger et al., 2014; Talwar and Hayatnagarkar, 2015). In most cases, these assays are primarily used for research purposes and are not considered part of the routine assessment of male infertility (Sikka and Hellstrom, 2016). This is mainly attributed to the lack of standardization and complexity of these assays in addition to the high costs, which adds a further financial burden to a couple undergoing fertility investigations (Talwar and Hayatnagarkar, 2015).

Therefore, several studies have sought to investigate the association between basic and advanced semen quality markers, however, results have often been found inconsistent. For instance, some studies have revealed that increased ROS production was negatively correlated with impaired sperm concentration, motility, morphology and viability (Agarwal et al., 2006; Aziz et al., 2004; Zorn et al., 2003). Other studies, however, failed to demonstrate any significant association between ROS levels and these semen parameters (Desai et al., 2010; Homa et al., 2015). Similarly, increased DNA damage was reported to be strongly correlated with sperm poor motility (Sheikh et al., 2008) whereas no such relationship was found in other studies (Karydis et al., 2005; Xia et al., 2004). Furthermore, a substantial association was observed between SOD activity and sperm concentration and motility (Badade et al., 2011; Siciliano et al., 2001), which seemingly contradict the results reported by Hsieh et al. (2002).

Few studies have further endeavoured to define the cut-off values for some modern semen parameters that could be useful in predicting the fertility potential of men. In this regard, Das et al. (2008) showed the cut-off value of $0.075-0.1 \times 10^6$ counted photons per minute (cpm)/10 million cells for ROS in the ejaculate is associated with higher fertilization outcomes. In another study, the seminal ROS cut-off value of 102.2 relative light units/sec (RLU/s)/million spermatozoa has also been suggested as a reliable indicator to differentiate between fertile and infertile men (Agarwal et al., 2015). Furthermore, López et al. (2013) demonstrated that levels DNA fragmentation above 25.5 % are associated with greater risk of failure *In vitro* fertilization (IVF) treatment, while relatively similar cut-off value of 26.1 % has recently been suggested (Wiweko and Utami, 2017).

In order to promote the diagnostic values of basic semen parameters, only one study has sought to investigate the relationship between basic and advanced semen markers with the aim of predicting sperm DNA fragmentation levels from the proportions of viability (Samplaski et al., 2015). Using Pearson's correlation coefficient, this study reported the threshold value of 30 % for DNA fragmentation to discriminate between semen samples with normal and compromised viability. That is, in samples with sperm viability ≥ 75 %, the DNA fragmentation was ≤ 30 %. According to their estimates, Samplaski, and colleagues calculated that the viability testing would successfully predict DNA fragmentation in nearly 67 % of men.

1.2 Motivation of the study

1.2.1 Aim I: Effect of abstinence period on semen quality.

The prevalence of male infertility is presently on the rise, therefore necessitating the need to find valid diagnostic approaches. According to the prescribed guidelines of the WHO, subjects must remain abstinent for a minimum period of 48 hours, but not longer than seven days prior to collecting a sample for a standard semen analysis (WHO, 2010). However, the basis for this recommendation remains contradictory, although there are indications in the literature that shorter abstinence periods might be beneficial. In addition, the overall quality of spermatozoa has been shown to be affected by the efficiency of storage in the epididymis and the rate at which spermatozoa pass from the proximal to the distal cauda region. Experimental studies have shown that moderate aging of hamster spermatozoa in the cauda epididymis was associated with a marked reduction in the ability of spermatozoa to undergo the acrosome reaction (Cuasnicu and Bedford, 1989). Similarly, experiments performed to compare the fertilizing capacity of rat spermatozoa recovered from different compartments of the epididymis, showed that spermatozoa sampled from the proximal cauda were significantly superior to those from the distal cauda region or vas deferens (Moore and Akhondi, 1996). In humans, semen collected from the same individual can display substantial variability, where changes have been observed between consecutive samples from the same donor (Keel, 2006). Therefore, combining the initial and sequential ejaculates collected within a very short period has recently been suggested as an effective approach for a potential shift of oligozoospermia patients towards the normozoospermic range (Bahadur et al., 2016a).

Chronobiological studies have furthermore shown that various changes occur in the body every 4 hours (Valsa et al., 2013). Despite this, no comprehensive study has been undertaken to investigate the effect of a short abstinence period, lasting only 4 hours, on conventional semen parameters, in addition to various existing sperm functional parameters such as acrosome reaction, sperm ROS and DNA fragmentation as well as seminal plasma antioxidant capacity. Additionally, it appears that no

study, as of yet, has comprehensively predicted basic and advanced variables of the second ejaculate (collected after a short abstinence period) from the core basic parameters obtained from the first ejaculate, which is collected after a long abstinence period. This could possibly assist in the decision making as to whether obtaining a second ejaculate after such a short period of abstinence would lead to better sperm quality and assisted reproduction outcomes in the case of a particular patient.

1.2.2 Aim II: Relationship between advanced and basic semen parameters

Aside from basic semen analysis, several biochemical and molecular investigations such as antioxidant assays, DNA fragmentation and ROS status have been employed to evaluate the functionality of spermatozoa in addition to providing valuable clinical insight into vital aspects of sperm functions. However, these advanced assays are highly complex, very expensive and lack universal standardization. Therefore, there is the need to develop a model that would assist in showing a relationship to indicate the extent to which changes in each individual measurement of semen analysis are related to changes in the advanced parameters. On the other hand, predicting advanced variables from a number of basic semen parameters will assist in eliminating the necessity for advanced sperm functional testing, representing cost-saving measures for some couples undergoing fertility assessment.

1.3 Outline of the study

Chapter 1 introduces the study with emphasis on a brief background, justification for the study, outlines of the entire thesis as well as the aims and the objectives of the study. Chapter 2 provides a short introduction to an extensive literature search, while the body of the literature review entails the processes involved in spermatozoa formation and development, specific attributes of spermatozoa and a number of factors that affect sperm quality. Chapter 3 is composed of protocols utilized in the study design and this proudly entails basic semen analyses in addition to various advanced techniques, such as DNA fragmentation, TBARS and antioxidant assays as well as various statistical tools used in the study. Chapter 4 entails a description of the statistically analysed results along with corresponding tables and figures. All the results are discussed comprehensively in Chapter 5. This includes interpretation, explanation and drawing of inferences in order to describe the findings of the study. This section is segmented based on the different aims and objectives of the study, while the conclusion and recommendations are captured in Chapter 6. The appendix contains addenda and supplementary data that may not be accommodated in any of the six Chapters, but is considered very important for the sake of detail and completeness.

As part of the study an in depth systematic review was conducted, which has been accepted for publication in the International Journal of Fertility and Sterility (Ayad et al., 2018). Furthermore, the original findings of this project specifically relating to the changes in parameters between short and long abstinence periods were also accepted for publication in the Middle East Journal of Fertility and Sterility (Ayad et al., 2017). These manuscripts form part of the Appendix.

1.4 Aims and objectives

Aim I:

The first aim of this study was to determine the effect of short (four hours) and long (four days) ejaculatory abstinence periods on semen and sperm quality.

Objective 1: Determination of the influence of short and long ejaculatory abstinence periods on basic semen parameters.

Objective 2: Determination of the influence of short and long ejaculatory abstinence periods on advanced semen parameters.

Objective 3: Prediction of both basic and advanced parameters of the second ejaculate from a set of basic semen parameters obtained from the first ejaculate.

Aim II:

The second aim of this study was to determine the relationship between basic and advanced semen parameters.

Objective 1: Correlations between basic semen parameters and advanced semen parameters.

Objective 2: Establishing cut-off values for the advanced semen parameters using a set of basic semen parameters with WHO defined reference values.

Chapter 2: Literature Review

The purpose of this chapter is to outline and discuss the current information available in the literature relating to this research. This section reviews information regarding the processes involved in spermatogenesis as well as basic and advanced sperm characteristics in addition to a number of factors that have been shown to influence sperm quality. This extensive review will allow for sound interpretation of the results and enable informed decisions with regards to methodology employed during the study and discussion of findings. The subsequent section will start with a broad revision of the steps involved in sperm production and development.

2.1 Sperm development/spermatogenesis

Spermatogenesis is a highly orchestrated series of events through which the immature diploid spermatogonia develop into mature haploid spermatozoa over an extended period of time (Hess and de Franca, 2009). The process of spermatogenesis is not activated until puberty and is then sustained for the remainder of life in normal men (Sharpe, 2010). Spermatogenesis occurs within the germinal epithelium of the seminiferous tubules (Figure 2.1), where the germ cells are sequentially organized into several layers from the base membrane towards the lumen. Each stage is morphologically distinct and is identified according to the cellular associations (Figure 2.2) observed in the tubular cross section. In humans, the entire process of spermatogenesis from the earliest stage of production to the ejaculation of spermatozoa is estimated to take an average of 64 days (Amann, 2008; Hess and de Franca, 2009; Misell et al., 2006).

During the embryonic development, the primordial germ cells transfer into the testis to be developed into immature germ cells named spermatogonia. At puberty, the spermatogonia undergo a series of mitotic divisions for renewing the stem cell and meiotic divisions for the production of spermatozoa (Amann, 2008). In men, two main types of spermatogonia have been identified; Type A comprises the stem cell population, and Type B which divides mitotically to yield primary spermatocytes. Type

A is the most rudimentary and is subcategorized into pale Type A and dark Type A spermatogonia (Durairajanayagam et al., 2015).

2.1.1 Mitosis:

Mitosis is a precisely regulated process, which involves the proliferation and maintenance of spermatogonia (Type A and Type B) and primary spermatocytes. Pale Type A spermatogonia divide mitotically to replenish themselves, as well as to differentiate into Type B spermatogonia for further development (Ehmcke and Schlatt 2006). Type B spermatogonia are the last germ cells to divide mitotically, producing preleptotene primary spermatocytes, which are the initial cells of the second phase. In men, about four generations of spermatogonia are prerequisite for the formation of preleptotene spermatocytes from a single spermatogonial stem cell. Preleptotene spermatocytes pass the tight junctions, moving away from the base of the seminiferous tubule (Hess 1999).

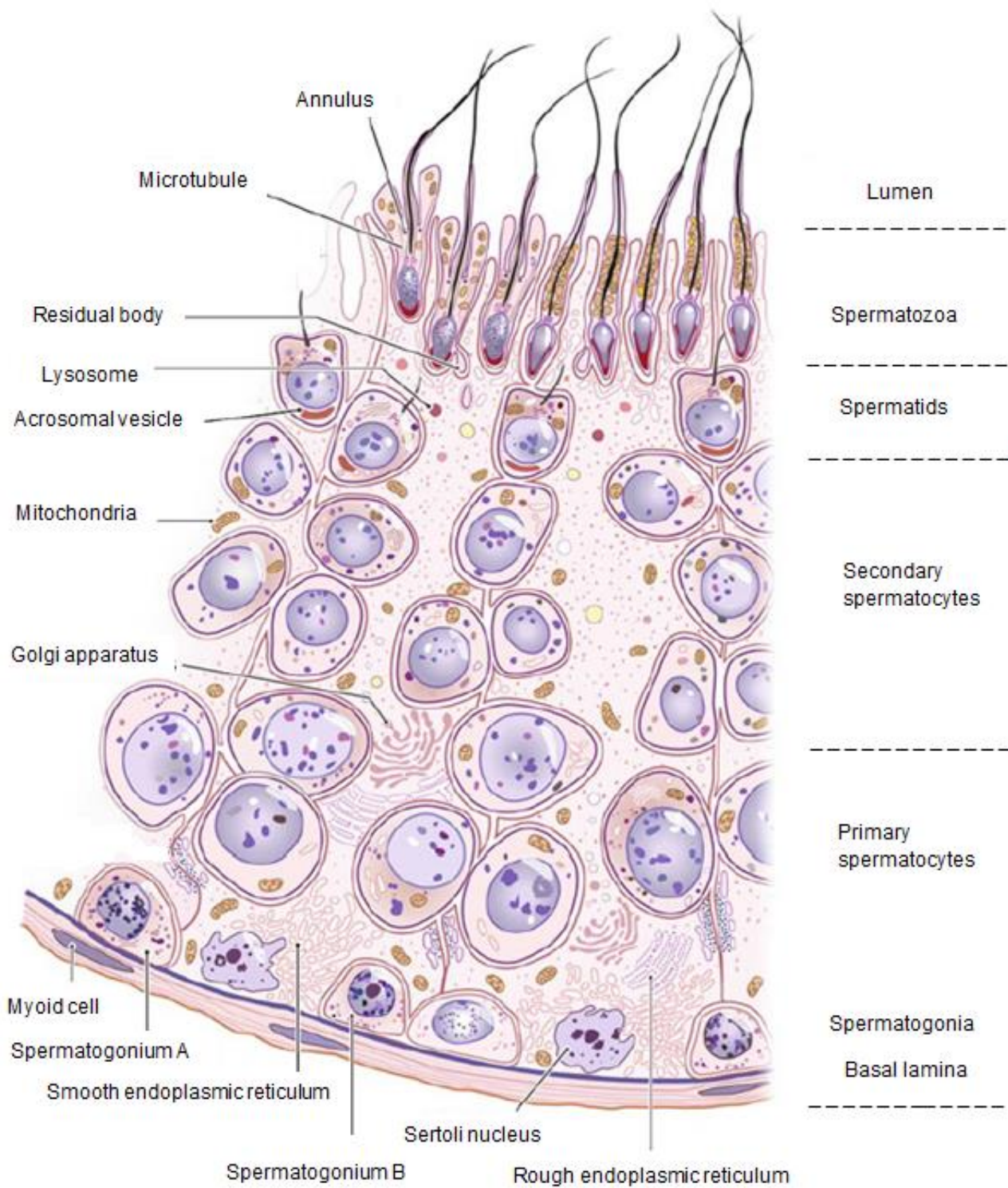


Figure 2. 1: Illustration of the major events in human spermatogenesis (Sharma and Agarwal, 2011).

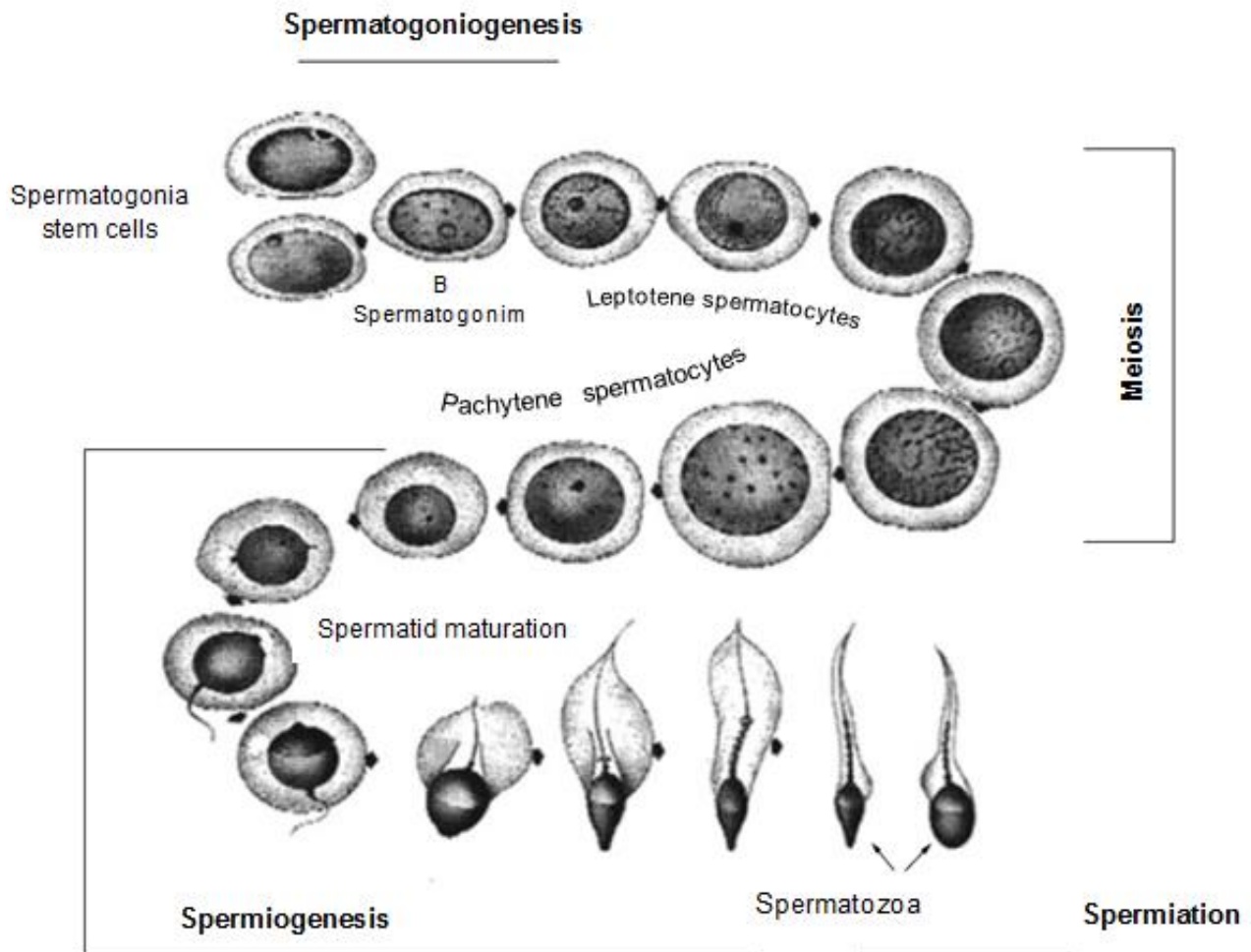


Figure 2. 2: Schematic representation of the main cell types that occur in the human seminiferous epithelium (Weinbauer et al., 2010).

2.1.2 Meiosis:

The meiotic process is a critical event in spermatogenesis as it involves a chromosomal exchange of genetic materials and a development of haploid cells with one set of chromosomes called spermatids (Weinbauer et al., 2010). Meiosis occurs in two successive divisions. The first meiotic division involves the transformation of primary spermatocyte into secondary spermatocyte that undergoes the second meiotic division to form four haploid spermatids. Each round of meiotic division consists of prophase, metaphase, anaphase, and telophase. Prophase of the initial meiotic division continues for three weeks, while the rest of the first meiosis and the entire second meiosis are accomplished within one to two days (Hess, 1999; Weinbauer et al., 2010). The end products of the telophase of the second meiotic division are round mitotically inactive spermatids. These cells have haploid chromosomes

(23X or 23Y) and remain lightly attached by fine links and enter the next phase of synchronous of differentiation and morphogenesis known as spermiogenesis (Sharma and Agarwal, 2011).

2.1.3 Spermiogenesis:

The round haploid spermatids derived from the second meiotic division undergo crucial developmental transformations leading to the formation of differentiated elongated spermatids and spermatozoa (Weinbauer et al., 2010). These changes include the appearance of the acrosomic granule in a close contact with the nuclear membrane, which subsequently flattens and caps about 1/3 of anterior surface of the nucleus. The nucleus also becomes flattened and further elongated with an increased state of chromatin condensation. During the maturation phase, a large portion of the cytoplasm is extruded as residual bodies. Another important event is the formation of the mitochondrial sheath and the dense outer fibres, which comprise the flagellum (Muciaccia et al., 2013, Sharma and Agarwal, 2011). Elongating spermatozoa remain connected to Sertoli cells until the spermiation; “the process by which mature spermatids are released from Sertoli cells into the seminiferous tubule lumen prior to their passage to the epididymis” (O'Donnell et al., 2011). Residual bodies retained in the immature spermatozoon are finally phagocytosed by Sertoli cells during the process of spermiation (Amann, 2008).

2.1.4 Epididymal maturation

On leaving the testis, the spermatozoon is morphologically normal, but immotile and unable to reach and fertilize an oocyte. Post-testicular maturation of spermatozoa is essential for the acquisition of progressive motility and fertilizing ability (Dacheux and Dacheux 2014). The epididymis is generally divided into three distinct anatomical regions; caput, corpus, and cauda. Testicular fluid, in which spermatozoa are transported, is reabsorbed almost entirely in the caput, leading to a remarkable increase in the concentration of spermatozoa by up to 100 fold. Fluidity of the sperm membrane is known to be a function of its fatty acid and cholesterol profiles. As the sperm transits from the caput to the cauda epididymis, its membrane fluidity increases gradually due to a progressive decline in the cholesterol/phospholipids ratio, which corresponds to the acquisition of progressive motility of the spermatozoa (Lindenthal et al., 2001). The ability of spermatozoa to acquire progressive motility develops gradually from the corpus to the caudal epididymis. Most spermatozoa retrieved from the caput are immotile or show only irregular curvature of flagella, but not progressive motility. This is possibly due to low intracellular cyclic adenosine 3', 5' monophosphate (cAMP) concentrations and/or high membrane rigidity (Dacheux and Dacheux 2014; Gatti et al., 2004).

In addition to the changes in the membrane compositions, spermatozoa passing through the epididymis undergo a series of extra biochemical and functional changes. These include changes in the expression of cell surface antigens, an increase in total surface negative charges as well as immunoreactivity and adenylate cyclase activity. These modifications are thought to be collectively involved in the acquisition of fertilizing ability of spermatozoa (Stoffel et al., 2002; Sullivan et al., 2005).

2.1.5 Storage of spermatozoa in the epididymis

Almost half of all spermatozoa die and disintegrate after leaving the testis and are reabsorbed by the epididymal epithelium. Nearly 70 % of the residual mature spermatozoa are reserved in the cauda epididymis, allowing for repetitive fertile ejaculations. The vas deferens is not a physiological site

for storage of spermatozoa, consisting only 2 % of the total spermatozoa present in the male tract (Mortimer, 1994; Sharma and Agarwal 2011). The storage capacity of the male tract is broadly determined by the volume of the cauda epididymis which provides a unique environment with slightly lower temperature than the testis that keeps the metabolic rate of the sperm minimised (Bedford 1978). In bulls and stallions, the number of stored spermatozoa in cauda epididymis is adequate for more than ten successive ejaculates (Sullivan et al., 2005), whereas in humans, less than three ejaculates of sperm are stored in the relatively poorly developed cauda epididymidis (Frenette, 2006; Sullivan et al., 2005).

Although specialized for sperm storage, the caudal environment is vulnerable to various physiological changes, and the functional status of the caudal spermatozoa cannot be maintained permanently. Prolonged sexual inactivity is associated with substantial changes in the senescent caudal spermatozoa, which eventually disintegrate. The relative participation of these spermatozoa to the subsequent ejaculation impairs semen quality, unless they are ejaculated and removed from the male tract at regular intervals (Mortimer, 1994).

2.2 Structure of human spermatozoa

Creation of the zygote (diploid cell) necessitates the combination of the haploid pronuclei from both male and female. The principal function of the spermatozoon is to deliver the male pronucleus to the receptive oocyte. To fulfil this, the spermatozoon must be able to preserve its DNA, transport it to the site of fertilization, and recognize and bind to the oocyte (Curry and Watson, 1995). The unique structural characteristics of the spermatozoon are crucial for its role in the fertilizing process. The normal mature spermatozoon is a particularly elongated cell, about 60 μm in length. It can be divided into four distinct regions namely, the head, the neck, the midpiece and the tail, which all are surrounded by a plasma membrane. The plasma membrane surrounding the spermatozoon is characterized by heterogeneous regional domains, each with different composition and surface antigen distribution that reflects specialized functions. The surface of the sperm head is divided into

the acrosomal region and the post acrosomal region. The acrosomal region is subdivided into the anterior acrosomal domain, involved in the acrosome reaction, and the posterior acrosomal domain that enables the sperm-egg membranes attachment and fusion (Toshimori, 2009). The structural characteristics of the spermatozoon, as viewed by transmission electron microscopy, will be described in the following section.

2.2.1 The head

The sperm head is bilaterally flattened and oval in appearance, measuring about 4.5 μm long, 3 μm wide and 1.5 μm thick (Figure 2.3). Most of the head is occupied by the nucleus and the acrosome, with a small amount of cytoplasm enclosed within the expanse of the plasma membrane (Maree et al., 2010). The acrosome, a vesicular like structure, caps about two thirds of the anterior region of the nucleus. The acrosome is composed of two membranes, the inner acrosomal membrane overlying the nucleus and the outer acrosomal membrane underling the plasma membrane. Between these two layers lies the acrosomal enzyme matrix. A network of cytoskeletal structures is distributed throughout the sperm head, particularly in the sub-acrosomal layer, between the inner acrosomal membrane and the nuclear envelope, as well as in the post-acrosomal space between the nucleus and the plasma membrane. Both of these two layers form the perinuclear theca, which covers most of the sperm nucleus excluding the implantation fossa, the narrow zone around attachment of the tail (Eddy and O'Brien, 2006). Dislocation of the implantation fossa of the nucleus has been shown to be an important contributor to the pathogenesis of flagellar coiling (Ricci et al., 2015). At the junction of the postacrosomal region of the head and the connecting piece is the posterior ring (Eddy and O'Brien, 2006).

The nucleus of a mature human spermatozoon is highly condensed and much smaller in size compared to that of a somatic cell. It is oval and flattened in shape, slightly concave anteriorly and convex posteriorly, and have an implantation cavity from which the flagellum originates. The sperm nucleus is bounded by a nuclear envelope consisting of two lipid bilayers, 7-10 nm apart, with a complete absence of nuclear pores (Eddy and O'Brien, 2006). As a result of the meiotic divisions during

spermatogenesis, the sperm nucleus is haploid and contains only a single set of 23 chromosomes. Nuclear DNA within spermatozoa is entirely inactive and remains in this state until its protamines are displaced upon entry into an oocyte. Mature spermatozoa do not have the capacity to synthesize RNA due to the lack of structures like ribosomes and nucleoli. The subsets of untranslated RNA found in ejaculated spermatozoa is assumed to be synthesized during spermatogenesis (Grunewald et al., 2005).

2.2.2 The neck

The sperm neck or connecting piece is a short linking segment and defines the boundary between the sperm head and the middle piece. The connecting piece is composed primarily of two main structures, the capitulum and the segmented columns. The capitulum is a dense fibrous plate-like structure attaches the basal plate, lining the implantation fossa, thus supporting the head-flagellum attachment. Extending to the posterior extreme of the capitulum are segmented columns, which branch into two major and five minor segmented columns. At their distal ends, these columns fuse to the nine longitudinal outer dense fibres extending throughout the length of the flagellum, thereby providing the flagellum with rigidity and structural support (Eddy and O'Brien, 2006; Ricci et al., 2015).

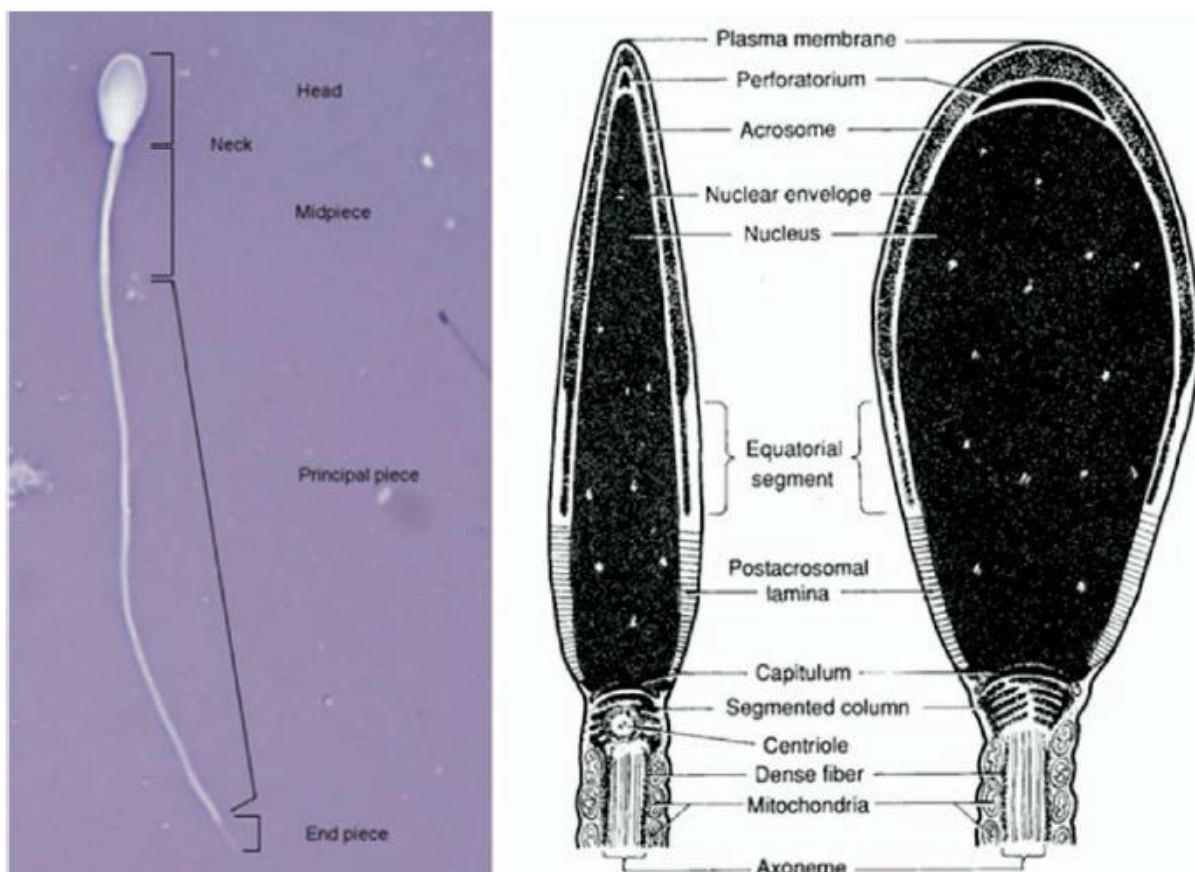


Figure 2. 3: Structure of the human sperm. Adapted from Brito 2007.

The connecting piece also comprises a pair of centrioles (proximal and distal). The proximal centriole lies beneath the basal plate, perpendicular to the long axis of the nucleus. The distal centriole stands parallel to the long axis of the sperm underneath the proximal centriole (Sathananthan et al., 1996). In mature spermatozoa, the distal centriole is virtually degenerated after the development of the axoneme, whereas the proximal centriole is involved in the production of a short microtubular structure known as the microtubule adjunct (Manandhar et al., 2000).

2.2.3 The midpiece

The midpiece of the spermatozoon is slender and $< 1 \mu\text{m}$ in width; its length is about 1.5 times the length of the head (WHO, 1999). It extends from the caudal end of the connecting piece to the annulus; a ring-like structure separates the midpiece from the principle piece of the flagellum. The annulus acts as a gated diffusion barrier that restricts the movement of particles between the two

domains (Curry and Watson, 1995). It also seems to stabilize the midpiece and prevents its mitochondria from slipping backwards. The midpiece consists primarily of the mitochondrial sheath, about 80 nm thick, lying directly underneath the plasma membrane. This sheath is a highly packed spiral arrangement of elongated mitochondria joined end to end around the underlying axoneme. The mitochondrial sheath is reputed to be an important source of energy essential for sperm movement (Briz and Fabrega, 2013). The central axis of the midpiece is occupied by the axoneme, which is surrounded by nine keratin-like protein fibres known as the outer dense fibres. The axoneme and the dense fibres of the midpiece extend to the distal tip of the flagellum (Toshimori and Eddy, 2014).

2.2.4 The tail

The flagellum of the human spermatozoon, about 45 μm in length, is slightly thinner than the midpiece, uniform, straight and uncoiled (WHO, 1999). The longest part of the flagellum is the principal piece, measuring about 40 μm . This segment plays an essential role in the generation of motive force for sperm motility. In addition to the axoneme and the outer dense fibres, the principal piece also comprises the fibrous sheath, which replaces the mitochondrial sheath of the midpiece. The fibrous sheath is a cytoskeletal structure underlying the plasma membrane and is composed of two longitudinal columns joined by circumferentially oriented ribs. The fibrous sheath is thought to provide the flagellum with elasticity and support. It also seems to modulate the beating characteristics of the flagellum, probably by restraining the degree of its bending. The short terminal piece of the flagellum lacks the fibrous sheath and other cytoskeletal structure and contains only the axoneme delimited by the plasma membrane (Brito, 2007, Briz and Fabrega, 2013, Curry and Watson, 1995).

The function of the flagellum is to provide cell motility, which enables the spermatozoon for the active passage through the boundaries of the female reproductive tract to reach and penetrate the oocyte. The structural properties of the sperm typically reflect these fundamental functional requirements. Flagellar motility is generated by sliding of the axonemal microtubule doublets past each other, leading to the development of symmetrical propulsive waves that propagate along the tail for the linear progression (Mortimer, 1997).

2.2.5 The axoneme

As shown in Figure 2.4, the axoneme is a highly ordered structure that forms the core of the flagellum. It is composed of two central singlet microtubules connected to each other by linkages and surrounded by the central fibrous sheath. The central microtubules are also surrounded by an array of nine pairs of peripheral microtubules that extend through almost the entire length of the sperm tail (Toshimori and Eddy, 2014). Each microtubule doublet is made of two structures known as subunit A and subunit B. The subunit A is a complete microtubule which is circular in cross section and measures 26 μm in diameter; whereas the subunit B is incomplete and C-shaped in cross section (Nojima et al., 1995). The adjacent outer microtubule doublets are connected to each other by two large motor protein projections, dynein arms, extending from each subunit A of one doublet to the subunit B of the adjacent doublet, in a clockwise fashion. Dynein arms are described as inner or outer according to their position relative to the central pair of microtubules (Neesen et al., 2001).

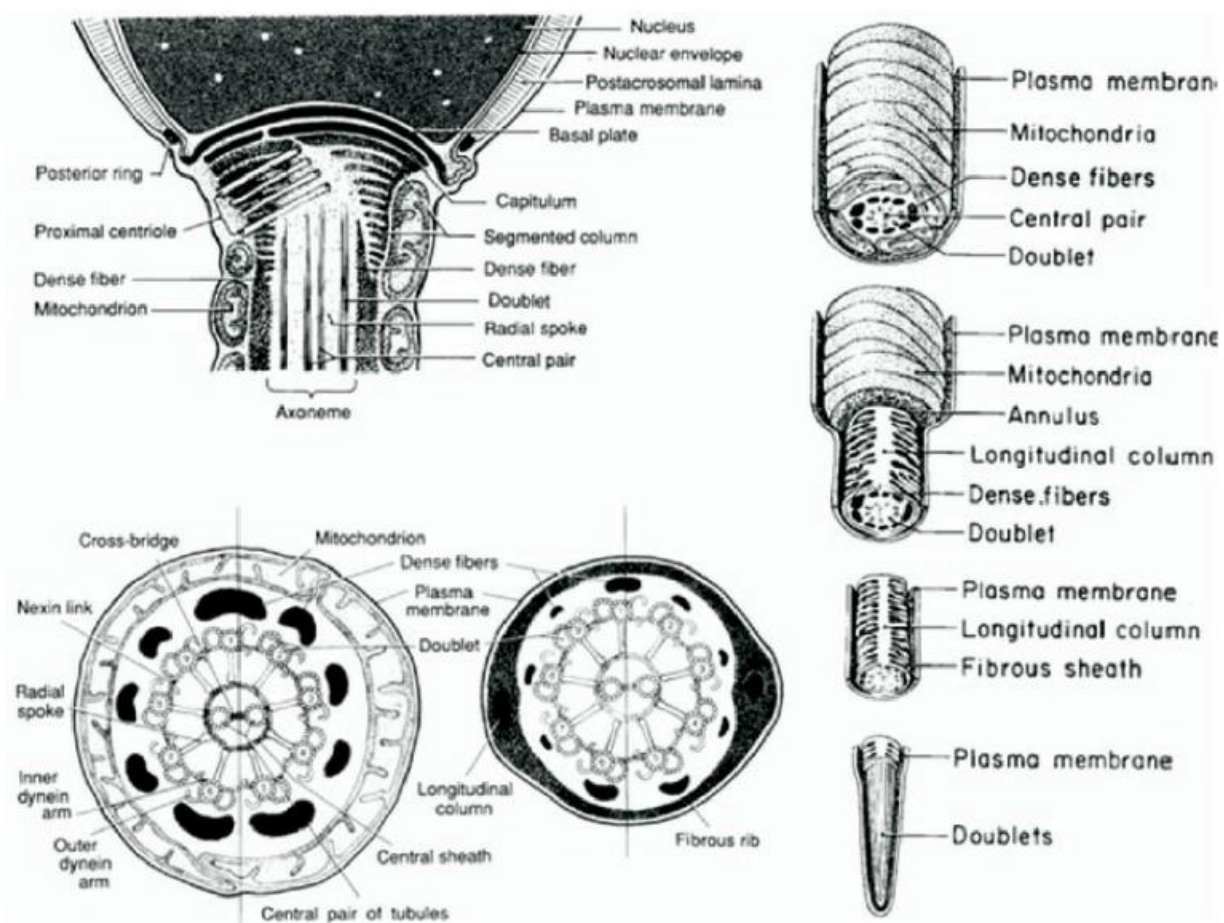


Figure 2. 4: Structure of the human sperm axoneme (Brito, 2007).

Both inner and outer dynein arms are assumed to contribute independently to the regulation of the flagellar waveform and the frequency of beating. Human spermatozoa lacking the outer dynein arms have been observed to swim in normal waveforms, but the beat frequency and the rate of progressive motility were significantly decreased compared with normal spermatozoa (Jouannet et al., 1983). By contrast, the absence of the inner arms has been associated with normal frequency of beating along with abnormal flagellar waveforms (Myster et al., 1999). The inner dynein arms are thought to play an essential role in the development and propagation of the flagellar bending motion throughout the generation of sliding forces between the adjacent peripheral microtubule. The outer dynein arms appear to maximize the microtubule sliding velocity, and consequently accelerating the flagellar beat frequency (Toshimori and Eddy, 2014).

Dynein possess ATPase activity responsible for the alteration of the chemical energy from ATP to the kinetic energy (Mortimer, 1997). The binding of the ATP to the dynein arm and its sequential hydrolysis results in a cyclical change in the angle of the dynein arms. This allows for repeated attaching and detaching of the cross-bridges of the dynein arm to the consecutive binding site along the length of the outer microtubule doublet in a regular pattern. This results in an active sliding motion of adjacent outer doublet microtubules relevant to each other leading to flagellar movement (Roberts, 2013).

An additional link between the neighbouring doublets is provided by nexin links. These links act as elastic elements, located infrequently at intervals of approximately 96 μm along the length of the microtubule doublets. Nexin links are thought to play a critical role in regulating the shear forces during doublet sliding, thereby retaining some degree of axonemal symmetry during sliding (Curry and Watson 1995). The digestion of the nexin links by elastase resulted in an increase in the bend angle, along with a decline in the beat frequency of the flagellum (Brokaw, 1980).

Microtubule A of the outer microtubules is further connected to the central fibrous sheath surrounding the two central singlet microtubules via cross linking projections known as radial spokes (Curry and

Watson 1995). During the microtubules doublet sliding, the radial spoke undergo cyclic attachment and detachment with the central pair complex (Huang et al., 1982). Mutant flagella with defective radial spokes have been shown to be capable of beating, but only in a symmetrical pattern. Thus, both central pair complex and radial spokes are possibly involved in transformation of simple symmetrical flagellar beating into the asymmetric motion, essential for the sperm to swim in a hyperactive manner (Smith and Yang 2004).

The dynein regulatory complex (DRC) is a crescent-shaped polypeptide lies adjacent to the site where nexin link is attached to the microtubule A, close to the base of the second radial spike. The RDC is positioned in the midway between the central pair, radial spokes, nexin linkages and the dynein arms (Roberts, 2013). This position enables it to transmit the local regulatory signals, which can be chemical or mechanical or both, between these structures (Ralston et al., 2006). Deterioration of the regulatory signals between these structures, as shown in DRC mutants, has been associated with a deficient control of flagellar beating, thereby abnormal bending wave patterns (Bower et al., 2013).

The nine outer dense fibres surrounding the flagellar axoneme are anchored to the caudal end of the segmented columns in the connecting piece, and extend along the middle piece and continuous for up to about 60 % of the principal piece of the human sperm flagellum. Each dense fibre is tightly attached to the adjacent peripheral microtubule doublet and numbered corresponding to the doublet to which it is attached (Toshimori and Eddy, 2014). Although their contribution to the active motility process remains uncertain, the outer dense fibres may play an important role in providing the flagellum with passive elastic properties to stiffen the axosomal dynein, thus lowering the maximum curvature of the tail (Petersen et al., 1999). They also provide the elastic recoil required subsequent to the sliding of the outer doublet microtubules past each other (Mortimer, 1997; Toshimori and Eddy, 2014).

2.3 Semen parameters

The accurate assessment of male fertility potential has long been an area of great interest for researchers and clinicians. Semen analysis remains the initial and the most important laboratory investigation for assessing male factor infertility. Results of semen analysis assist in determining the treatment approach and the subsequent strategies of sperm preparation in male infertility factor (Sharlip et al., 2002).

The subsequent section will briefly review the basic (conventional) parameters of semen analysis, which include semen viscosity, pH, volume, sperm concentration, sperm motility, morphology and viability.

2.3.1 Semen viscosity

According to the WHO Laboratory Manual for the Examination and Processing of Human Semen, semen viscosity is recommended among the parameters to be measured in the preliminary macroscopic examination of a semen sample (WHO, 2010). Under normal conditions, semen coagulates after ejaculation and gradually liquefies within 15-20 minutes. However, semen samples that do not fully liquefy and retain some viscosity for 60 minutes after ejaculation are considered hyperviscous (WHO, 2010). Semen hyperviscosity is considered a useful marker for the evaluation of secretory function of the seminal vesicles and prostate (Gonzales et al., 1993).

Although the exact reason of aberrant semen viscosity is unknown, hyperviscosity is largely attributed to male accessory gland dysfunction, infection and inflammation (Du Plessis et al., 2013). Hyperviscous seminal fluid is a condition associated with changes in the physical and chemical properties of the ejaculate, which detrimentally influence sperm function and its fertilizing efficiency (Elia et al., 2009). This condition is estimated to occur in nearly 12 to 29 % of semen samples and is rumoured to contribute significantly to the impairment of semen quality (Tadeu Andrade-Rocha, 2005; Wilson and Bunge, 1975). Hyperviscous semen has been found to have elevated levels of sperm antibodies, with higher proportions of morphologically abnormal spermatozoa (Moulik et al., 1989).

Hyperviscosity has also been associated with other abnormalities such as decreased sperm count (Esfandiari et al., 2008) and impaired motility (Elzanaty et al., 2004).

2.3.2 Semen pH

At the time of ejaculation, spermatozoa suspended in the epididymal fluid pass from their reserves in the cauda epididymis through the ductus deferens to be emitted through the penile urethra, which has already been lubricated by the secretions of Cowper's gland (Mortimer, 1994; Owen and Katz, 2005). Just prior to emission, the ejaculate is initially mixed with 0.5–1.0 mL of the prostatic secretion, consisting about 15 to 30 % of the entire volume of ejaculate. Prostatic secretion is a serious fluid with a slightly acidic pH ranging between 5.5–6.8 and. Finally, the ejaculate is mixed with approximately 4 mL of seminal vesicle secretion, a yellowish viscose alkaline fluid (pH 7.6–8.6). This contributes up to 70 % of the total ejaculate (Behre 2011; Mortimer, 1994). Accordingly, semen pH is a result of the balance between the pH values of various genital accessory gland secretions, in particular the acidic prostate fluid and the alkaline seminal vesicle fluid. The lower threshold value of semen pH in fertile men has been estimated to be 7.2 (WHO, 2010).

Semen pH is considered an important indicator of sperm fertilizing potential. The slight alkalinity of seminal fluid is essentially required to neutralise the acidic secretions of the vagina, which is detrimental to the sperm (Peek and Matthews, 1986). Acidic ejaculate could be a marker of the blockage of the seminal vesicles, whereas alkaline ejaculate is commonly associated with infections of the accessory glands. The assessment of pH in semen is especially important when evaluating the patients with azoospermia. In cases of congenital bilateral absence of the vas deferens and ejaculatory duct obstruction, the reduced semen volume is usually accompanied by acidic pH (Banjoko and Adeseolu, 2013; WHO, 2010).

Changes in pH may affect the metabolic rate, and thus alter several aspects of sperm quality. *In vitro* culture of human spermatozoa in acidic nutrient solutions (pH 5.2) resulted in a significant decline in sperm progressive motility, velocity and viability compared with that in solutions with pH 7.2. In

clinical trials, a substantial reduction in sperm concentration and motility was observed in patients with semen pH lower than the minimum WHO threshold value (Zhou et al., 2015). The detrimental effect of acidic pH on sperm quality might be attributed the decline in Na^+/K^+ -ATPase activity and calcium levels at acidic milieu, which consequently impairs sperm functions, including motility, capacitation and acrosome reaction (Zhou et al., 2015).

2.3.3 Semen volume

The lower reference value for semen volume as recommended by the WHO is 1.5 mL (WHO, 2010). Precise measurement of the semen volume is important as accurate concentrations of spermatozoa and non-sperm cells in the ejaculate are built on the initial determination of the volume (WHO, 2010). Semen volume has consequently been suggested to be an early indicator of impaired semen quality even before the identification of any abnormality in concentration, motility and morphology of spermatozoa (Pasqualotto et al., 2006). In addition to the value of pH, semen volume has been suggested to be a reliable indicator of the secretory functions of the accessory glands, particularly the seminal vesicles (Daudin et al., 2000).

The cellular component is made up mainly of spermatozoa, contributing only about 1-5 % of the total volume of the ejaculate. The bulk of ejaculated semen is a mixture of components provided mainly by secretions from the major male accessory reproductive glands, i.e. seminal vesicles, prostate gland, and Cowper's bulbourethral glands (Mortimer, 1994). An additional small volume is also provided by other accessory glands such as, ampullary, Littre and Tyson's glands. The first fraction of the ejaculate, approximately 5 %, originates from the bulbourethral or Cowper's gland and Littre glands, whereas the second fraction consists of prostatic secretions and comprises about 15 to 30 % of the total volume. Up to 70 % of the total ejaculate is supplied by seminal vesicles, and represents the last fraction of the ejaculate (Mortimer, 1994; Owen and Katz, 2005).

2.3.4 Sperm Concentration

The concentration of spermatozoa in semen, expressed as millions per millilitre, is a critical indicator of semen quality and a prognostic factor for fertility potential (Guzick et al., 2001; Nallella et al., 2006). Sperm concentration is not recommended as an accurate measure of the testicular sperm production since it is influenced by the genital accessory gland secretions in which the concentrated epididymal spermatozoa are diluted at ejaculation (WHO, 2010). The T.S.C. expressed as millions per total ejaculate, is obtained from the sperm concentration and suggested to be a better marker for the evaluation of the effectiveness of spermatogenesis (Amann, 2009; Ng et al., 2004).

2.3.5 Sperm viability

Evaluation of spermatozoal membrane integrity, assessed by viability testing, is an important determinant to evaluate and predict the fertilizing potential of spermatozoa. Declined levels of viable cells have been directly correlated with male factor infertility (Correa-Pérez et al., 2004). The lower reference limit for sperm viability is estimated to be 58 % (WHO, 2010). Sperm vitality is one of the routine assessments of basic semen analysis, and is especially recommended in samples where the percentage of motile spermatozoa is less than about 40 %. Viability testing can be useful in validating motility assessment, as the percentage of live spermatozoa should exceed that of the total motile cells (WHO, 2010). The viability status of the selected spermatozoon for intracytoplasmic sperm injection (ICSI) has to be precisely evaluated, as the only necessity for the success of the ICSI is the injection of a live spermatozoon (Nagy et al., 1995). Furthermore, a direct correlation has recently been proposed between sperm viability and the level of the DNA fragmentation, suggesting that the viability status may provide an indication of the DNA fragmentation outcomes of the ejaculated spermatozoa (Samplaski et al., 2015).

2.3.6 Sperm morphology

The morphological characteristics of spermatozoa have been considered to be of great value in the assessment of semen quality (Maree et al., 2010; Menkveld et al., 2011). To be considered

morphologically normal, the whole spermatozoon and its three distinct areas; the head, midpiece and tail must fit the Strict Criteria in terms of their size and shape (WHO, 2010). Defining a morphologically normal sperm, with potential fertilizing capability was achieved by observing spermatozoa recovered from the female genital tract, particularly in the post coital endocervical mucus and from the surface of the zona pellucida (WHO, 2010). Morphologically normal spermatozoa are expected to be a result of an uninterrupted spermatogenic process. The morphogenetic changes in human spermatozoa during spermatogenesis and/or epididymal maturation can be associated with imperfections and anomalies resulting in the production of spermatozoa with various abnormal forms, which can be identified in a routine semen analysis (Auger, 2010). Therefore, abnormal sperm morphology could provide an indication of testicular or epididymal impairment, which might be mediated by various biological and environmental exposures (Auger, 2010; Auger et al. 2001; Menkveld et al., 2011).

The most commonly used classification systems for sperm morphology are the WHO criterion and the Tygerberg Strict Sriterion. The Strict Criteria, as originally described by Kruger et al. (1987) uses more sensitive morphological analysis, in which the spermatozoon is categorized normal only if it falls within a strictly defined range, and the borderline forms are considered abnormal. A threshold of 14 % has been recommended as a normal value for morphology evaluated according to the strict criteria (Ghirelli-Filho et al., 2012). In its earlier editions, the WHO criteria suggested a total of 80 % or more normal forms for a semen sample to be considered normal (WHO, 1980). However, considering the morphometric information which were identified and introduced to the evaluation system over years, the latest and revised WHO guidelines minimised the lower cut-off value for normal forms to 4 % (WHO, 2010).

Sperm morphology has been suggested as the most important discriminatory parameter that differentiates between fertile and infertile men (Guzick et al., 2001). Furthermore, the proportion of spermatozoa with normal forms has been shown to be associated with higher success rates of fertilization (El- Ghobashy and West, 2003; Kihaille et al., 2003). Likewise, impaired ICSI results

have been reported in presence of small-headed (Kihale et al., 2003), Large-headed (Chelli et al., 2010) or elongated spermatozoa (Osawa et al., 1999).

Morphologically abnormal spermatozoa are possibly incapable of progressive movement towards the oocyte, as it may lack the machinery required to propagate within the female reproductive tract. In consonance, spermatozoa with normal forms have been found to have higher progressive motility accompanied with improved straightness and linearity compared with those with abnormal morphology (Love, 2011; Ma et al., 2006). In addition, the process of sperm-oocyte binding appears to be highly selective in terms of the morphological characterizations of the sperm. A number of studies have shown that morphologically abnormal spermatozoa, with a special focus on the acrosomal region, have a lower chance to bind to the zona pellucida (Garrett et al., 1997; Liu and Baker, 1992; Liu et al., 2003).

2.3.7 Sperm motility

Testicular spermatozoa of humans are often immotile or only exhibit slight motility. However, during the epididymal transit, spermatozoa undergo a series of significant maturational changes leading to the acquisition and development of motility. Full motility is then induced and displayed at the time of ejaculation when spermatozoa are mixed with the various accessory gland secretions (Mortimer, 1994).

Determination of the motility characteristics of ejaculated spermatozoa has been shown to be of the utmost importance for the prognosis of male fertility potential as it provides vital information about the functional competence of the sperm (Gunalp et al., 2001). While the T.S.C. is recommended as an accurate measure of the efficiency of spermatogenesis (Ng et al., 2004), the percentage of motile spermatozoa in the ejaculate provides an indication of the epididymal sperm maturation (Fàbrega et al., 2012). Motility is essentially acquired for the spermatozoa to migrate through the harsh environment of the female genital tract to the site of fertilization. Therefore, semen containing spermatozoa with lower proportions of normal motility is usually associated with failed fertilization

(Suarez and Pacey, 2006). Not only is motility necessary for transport, but changes in the flagellar motion also play an essential role at the site of fertilization where the mechanical driving force, generated by motility, help the sperm to reach the ovum and propel through its outer layers (Burkman, 1984).

The latest and revised WHO guidelines recommend a simplified grading system for the classification of motility that categorizes spermatozoa into three main motility groups. These groups include; progressive motility “spermatozoa moving actively, either linearly or in a large circle, regardless of speed”, non-progressive “all other patterns of motility with an absence of progression; e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed”, and immotile “no movement” (WHO, 2010). This categorization system represents a major change from the previous edition, in which each spermatozoon is categorized as being type a, b, c or d according to its individual motility characteristics. Type a spermatozoa are characterized by rapid progressive motility with a minimum speed of 25 $\mu\text{m/s}$ at 37°C, which is nearly equivalent to the movement of half a tail length or five head lengths distance in each second. Type b spermatozoa are also progressively motile, moving in a forward manner with a speed of 5 $\mu\text{m/s}$ or more, but still slower and more sluggish than type a ones. Spermatozoa with non-progressive motility are categorized as type c, which are weakly motile, unable to swim forward, and move in an irregular pattern at a speed less than 5 $\mu\text{m/s}$. Finally, spermatozoa that display no active tail movement are considered immotile and classed as type d (WHO, 1999).

Progressive motility is well recognised as being of particular clinical interest as the forward movement is essential for the passage through the female reproductive tract and for the success of the consequential interaction between the sperm and the oocyte. Progressive motility is prerequisite for the spermatozoon to penetrate the zona pellucida both *in vivo* and *in vitro*, and is thought to be a useful marker that reflects the ability of the spermatozoon to fertilize an egg (Simon and Lewis, 2011; Turner, 2005). Therefore, when reporting spermatozoal motility, it is recommended to clearly specify progressive motility or total motility (Franken and Oehninger, 2012). According to the latest WHO

guidelines, motility is considered normal when at least 40 % (38–42) of the total spermatozoa are motile, whereas the percentage of progressively motile cells is 32 % (31–34) or more (WHO, 2010).

2.3.8 Sperm kinematic movement

The automatic analysis of sperm motility by CASA instruments allows for the objective estimation of various parameters which depict certain kinematic measures of sperm movement (Kraemer et al., 1998; Mortimer and Mortimer, 1990). Kinematics is “time-varying geometric aspects of motion that are distinct from calculations of mass and force” (Drobnis et al., 1988). The CASA system identifies each individual sperm in the microscope field and capture a series of digital images of the spermatozoal head movement, so that their individual trajectories can be reconstructed and tracked (Mortimer et al., 2015). Three sperm kinematics are considered to be velocity measures, which are generally used to describe different aspects of sperm progressive motility (Figure 2.5); these include the curvilinear velocity (VCL), the straight-line velocity (VSL) and the average-path velocity (VAP) (Mortimer, 1997; WHO, 2010). VCL is a measure of the time-average velocity ($\mu\text{m/s}$) of the centroid of the sperm head along its actual curvilinear path. It is calculated by finding the total distance between first and last head points divided by the time elapsed. VCL formerly known as total swimming speed (Katz and Overstreet, 1979) and sperm head velocity (Suarez et al., 1983). VSL is a measure of the time-average velocity ($\mu\text{m/s}$) of the centroid of the sperm head along the straight-line trajectory between its first and last points. It is computed by finding the total distance travelled along the linear path divided by the acquisition time. VSL was originally termed net velocity (Stephens et al., 1988), velocity of progression (Mortimer et al., 1986), net displacement (Tessler and Olds-Clarke, 1985) and swimming speed (Ishijima et al., 1986). VAP is a measure of the time-average velocity ($\mu\text{m/s}$) of the centroid of the sperm head along the smoothed trajectory, which is constructed by averaging several points on the actual curvilinear path. It is computed by dividing the length of the smoothed track by the acquisition time. VAP was formerly called the position-averaged velocity (Stephens et al., 1988).

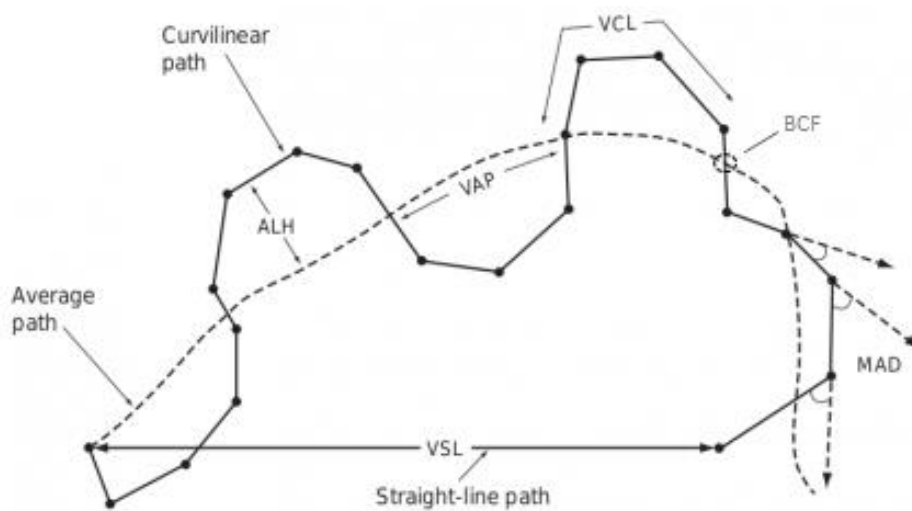


Figure 2. 5: The main kinematic measurements involved in sperm tracking. Modified from WHO, 2010.

From the above-mentioned velocity values, three velocity ratios have been derived, these are linearity LIN (%) which refers to the linearity of the curvilinear path and is calculated as $(VSL/VCL) \times 100$, it was previously described as a progressiveness ratio. Straightness STR (%) measures the linearity of the average path and is calculated as $(VSL/VAP) \times 100$, the STR was formerly known as linear index (Stephens et al., 1988). The Wobble WOB (%) measures the magnitude of the oscillation of the actual path around the average path, formerly called curvilinear progressiveness ratio and is calculated as $(VAP/VCL) \times 100$ (Mortimer and Mortimer, 1990; Samuels and Van der Horst, 1986; Stephens et al., 1988).

The amplitude of lateral head displacement (ALH) measures the degree of lateral displacement of the sperm head's centroid around its average path (μm). The ALH can be determined mathematically by measuring the length of the risers, which are straight lines extending between each point on the average path and its corresponding point on the actual curvilinear path. The ALH value can be calculated from the maximum riser value, which is then doubled to give the track-maximum measurement (ALH_{max}), or from the averaged riser values to be doubled and expressed as the track-average measurement (ALH_{mean}) (Mortimer, 1994).

Beat/cross frequency (BCF) indicates the frequency (hertz [Hz]) with which the curvilinear path crosses the average path; such crossovers occur two times within each flagellar beat cycle. Therefore, BCF is considered a measure of the flagellar beating frequency, as a new flagellar beat is initiated once the actual sperm trajectory crosses the average path. BCF provides further indication of the frequency of the rotational movement of the sperm head around its longitudinal axis of progression, providing the sperm head rotates by 180 degrees at the peak of each lateral displacement with each beat initiation (Mortimer, 1997).

As a frequency measurement derived from the curvilinear and the average tracks, the BCF value is largely dependent on the frame rate and has been shown to decline with reducing frame rates (Mortimer et al., 1988). When the frequency of the event being measured is above the “Nyquist” number, which is equivalent to one-half the image sampling frequencies, those events will probably not be represented sufficiently and the profile of the signal processed will eventually be aliased. The mathematical calculation of BCF can apparently be further confounded by the incidence of asymmetrical trajectories (Owen and Katz, 1993; Mortimer and Swan, 1999).

Changes in the direction of the sperm head motion along its actual track can be calculated as angles and be used to characterize the overall trajectory of the sperm cell. Mean angular displacement (MAD) is a measure of the curvature trajectory of spermatozoa, and defined as “the time average of absolute values of the instantaneous turning angle of the head along its curvilinear trajectory” (Boyers et al., 1989). Two angle parameters have also been used to measure the angular displacement of sperm head from linearity. The absolute angle refers to the mean value of each triplicate of points along the average trajectory without consideration of the direction of the movement, the algebraic angle is also reckoned in a similar way, but the right or left deviations are indicated as positive or negative values. The produced mean angular values for the sperm moving in a linear pattern are essentially lower than those values produced for those deviating from the straight line (Stephens et al., 1988).

The choice of sperm preparation method appears to have a direct effect on the predictive value of the sperm kinematic variables assessed by CASA in relation to fertility potential. For instance, in fresh

ejaculates, significant correlations were demonstrated between ALH, VCL and VSL and fertilization rates. However after swim-up separation, STR was shown to be the only predictor of achieving pregnancy after IVF treatment compared with other semen variables (Hirano et al., 2001). By contrast, no significant association was found between CASA kinematic parameters and the pregnancy rates after intrauterine insemination (IUI) when the semen samples were analysed after thawing and density gradient preparation (Freour et al., 2009).

2.3.9 Sperm hyperactivity

The pattern of sperm motility has been shown to change from progressive to hyperactivated during the final stage of the capacitation process (Goodson et al., 2011). Hyperactivated motility in spermatozoa has initially been reported in the golden hamster as an extremely vigorous pattern of flagellar beating observed after incubation with follicular fluid (Gwatkin and Andersen, 1969; Yanagimachi, 1970). It was subsequently demonstrated in human spermatozoa during *in vitro* capacitation (Burkman, 1984; Mortimer et al., 1997). Variations in the swimming patterns between hyperactivated and non-hyperactivated spermatozoa are basically determined by differences in the degree of the axonemal bending as well as the propagation rate of the beats along the length of the flagellum (Mortimer, 1997). The highly vigorous motion of hyperactivated spermatozoon is considered to be a result of two main types of extremely high curvature of flagellar bends. The first bending is not propagating and localised at the proximal region of the flagellar midpiece allowing the head to twist in a hatchet-like curve. By contrast, the second bending occurs in the distal region of the midpiece and propagates as a wave down the length of the flagellum producing an eight-like trajectory (Kay and Robertson, 1998). Hyperactivated spermatozoa have been shown to swim with greater flagellar flexibility which allows them to turn around and escape from the pockets of the mucosal folds through the lumen of the oviduct (Suarez, 2004). The asymmetric flagellar bending during hyperactivation might also assist in the detachment of the spermatozoal head from the end salpinx of the caudal isthmus, a portion of the Fallopian tube where the spermatozoa are supposed to be reserved and subsequently released to continue their journey towards the site of fertilization

(Hunter, 2011; Pacey et al., 1995). Moreover, hyperactivity associated changes in the swimming pattern enable the spermatozoon to migrate more effectively through thick viscoelastic mucus of the oviduct lumen and to penetrate the cumulus oophorus and then the zona pellucida surrounding the oocyte (Suarez, 2004). Recently, hyperactivation has been suggested to play a critical role in the chemotactic response of spermatozoa to substances released from the matured oocyte and the surrounding cumulus cells, so that they are sharply reoriented toward the direction of the chemoattractant gradient (Armon and Eisenbach, 2011). Therefore, due to its various physiological advantages in human spermatozoa, hyperactivated motility has been proposed as a biomarker that can be used, in combination with other sperm functional tests, to estimate the fertilization potential of human spermatozoa. In this regard, available evidence indicates that stimulation of sperm hyperactivity may be of great value in promoting *in vitro* fertilization success rates (Breznik et al., 2013).

The physiological bases of hyperactivation remain poorly understood (Mortimer et al., 2015). However, the regulation of sperm hyperactivation *in vivo* appears to be influenced by various components of the internal environment of the female genital tract, including the oocyte and its surrounding follicular cells and secretions (Kay and Robertson, 1998; Suarez, 2004). *In vitro*, hyperactivation has been shown to be induced by incubating the spermatozoa in supplemented laboratory media. In humans, the proportion of spermatozoa with hyperactive motility induced with this approach is estimated to reach about 20 %, while this rate has been shown to rise as the incubation period increases (Ooi et al., 2014). Several different physiological and chemical factors have been used to stimulate hyperactivation *in vitro*, such as bovine serum albumin (Yanagimachi, 1970), potassium (Fraser, 1983), bicarbonate (Neill and Olds-Clarke, 1987), progesterone (Mbizvo et al., 1990), calcium (Suarez et al., 1993), as well as glucose (Williams and Ford, 2001).

The advanced semen parameters performed in this study, which includes acrosome reaction, DNA fragmentation, ROS, lipid peroxidation and antioxidant analyses, will be described in the following section.

2.3.10 Acrosome reaction

In vivo, ejaculated spermatozoa do not have the capacity for fertilization until they undergo series changes in the cellular metabolism and the physicochemical properties of the plasma membrane (Patrat et al., 2000). These modifications are collectively known as capacitation. Capacitation is a maturation process essential for the acrosome reaction to occur at the zona pellucida subsequent to the sperm attachment. This process involves alterations in the plasma membrane fluidity due to the removal of cholesterol and changes in the amount and distribution of membrane glycoprotein and phospholipid contents. Sperm capacitation is also associated with an increase in intracellular Ca^{2+} ion concentration and hyperpolarization of the membrane. Various agents such as; oviductal and follicular fluids, progesterone, serum albumin and zona pellucida glycoproteins have been shown to induce acrosomal exocytosis in human spermatozoa (Gupta and Bhandari, 2011; Mansour et al., 2008; Patrat et al., 2000).

The acrosome reaction is a stimulus-secretion coupled exocytotic process characterized by the fusion between the external acrosomal membrane and the plasma membrane (Breitbart et al., 1997). This eventually leads to the release of various hydrolytic and proteolytic enzymes such as; hyaluronidase and acrosin, leaving the frontal region of the sperm head covered by only the inner acrosomal membrane. The released chromosomal enzymes digest a path through the zona, which enables the sperm to penetrate the oocyte. However, the true acrosome reaction occurs in live and intact spermatozoa and leads to fertilization must be discriminated from the vesiculation of the acrosomal membrane and the overlying plasma membranes, which commonly reflects the cell death (Avdatck et al., 2010).

The physiological acrosome reaction is an irreversible event that must occur at an appropriate time, thereby the spermatozoon must continue to have an intact acrosome until it binds to the zona pellucida (Green et al., 1999). Premature acrosome reaction results in the loss of the sites on the sperm head required for the recognition and binding of the spermatozoon to the zona pellucid (Liu and Baker, 1992). In samples with normal seminal parameters, the majority of spermatozoa exhibit intact acrosome whereas only a small fraction of the spermatozoa may display spontaneous acrosome reaction despite the absence of any stimulants, which is deemed to be of no clinical relevance (Esteves et al., 2015). After capacitation, the percentage of induced acrosome-reacted spermatozoa increases significantly up to 40 % (Esteves and Verza, 2011). The total percentage of acrosome-reacted spermatozoa represents a combination of induced acrosome-reacted and spontaneously reacted spermatozoa (Zeginiadou et al., 2000). In general, infertility has been associated with higher levels of spontaneous acrosome reactions and lower levels of induced reactions (Henkel et al., 1993; Liu et al., 2001; Pampiglione et al., 1993).

Although it is not commonly practiced in laboratories and remains a research interest, assessment of the acrosomal status represents one of the most important approaches to evaluate the sperm's fertilizing ability (Esteves et al., 2007). Available assays for evaluating the acrosomal status in the ejaculate have been developed in two ways; either by estimating the percentage of spontaneously acrosome-reacted spermatozoa or by assessing the ability of spermatozoa to initiate acrosomal exocytosis when exposed to an appropriate stimulus i.e. zona pellucida and progesterone (De Jonge, 1994). Various staining techniques are available for the assessment of the acrosome integrity of human spermatozoa. These techniques are commonly based on the use of dyes or fluorescent markers to discriminate between acrosome-reacted and acrosome-intact sperm cells. The most frequently used method for assessing the acrosomal status involves the labelling of specific regions of the live sperm with a lectin (e.g. the pea derived *Pisum sativum* agglutinin [PSA] or the Peanut Agglutinin [PNA]) conjugated with a fluorescent probe (e.g. fluorescein isothiocyanate [FITC]). The PSA binds to α -mannose and α -galactose moieties associated with the acrosomal matrix, whilst the PNA displays

more specific affinity to bind β -galactose moieties which is associated typically with the outer acrosomal membrane. The acrosomal status can then be assessed by fluorescent microscopy or flow cytometry, with the disappearance of the label indicating acrosomal exocytosis (Graham, 2001; Hamed et al., 2014; De Jonge, 1994; Patrat et al., 2000).

Acrosome defects, malformations and dysfunctions have been associated with inhibited fertilizing capacity of the spermatozoa (Schill, 1991). An increased spontaneous acrosome reaction has also been linked with unexplained male infertility, apparently due to the inability of prematurely-reacted spermatozoa to release their acrosomal contents when exposed to proper stimuli (Esteves et al., 2015). Decreased responses to acrosome inducers have generally been suggested to contribute to impaired fertilization rates after IVF (Fenichel et al., 1989; Pampiglione et al., 1993).

The ability of spermatozoa to undergo acrosome reaction is thought to develop during the epididymal transit. The failure to undergo this reaction may indicate impaired epididymal function (Boué et al., 1996; Haidl et al., 1994). Acrosomal status has also been strongly associated with several abnormalities in sperm parameters. For instance, various morphological defects (e.g. globozoospermia; round-headed spermatozoa lacking acrosome) have been related to lower proportions of inducible acrosome-reacted spermatozoa (Carrell et al., 1994; Dam et al., 2006; Parinaud et al., 1995). Sperm motility has been reported to decline considerably after the acrosome reaction has been undergone. This is supported by the significantly lower proportions of induced acrosome reaction observed in asthenozoospermic samples compared with normozoospermic samples (Pilikian et al., 1992). Similarly, the percentage of progressive motility has been shown to decrease in sperm populations with high spontaneous rates of acrosome-reaction (Moutaffian and Parinaud, 1995).

2.3.11 Sperm DNA fragmentation

Conventional semen analysis, when performed under strict methodological criteria, remains the cornerstone for the assessment of the male fertility potential (Evgeni et al., 2014; WHO, 2010). These

tests are crucial to obtain the important information on which clinicians base their preliminary diagnosis (Giwerzman et al., 2010). However, the parameters of the conventional semen analysis have a relatively limited power to determine the probability of conception (Oleszczuk et al., 2013). Approximately 25-40 % of infertile males are estimated to have normal semen analysis profiles (Bungum et al., 2011; Gudeloglu et al., 2015; Van der Steeg et al., 2011). To avoid these limitations, a range of new sperm quality parameters have been developed (Erenpreiss et al., 2006).

Spermatozoal DNA integrity has been recognised as a prerequisite for normal fertilization and proper transmission of genetic information (Evgeni et al., 2014). The assessment of sperm DNA integrity along with routine semen analysis provides further valuable information about the quality of the sperm as well as the pregnancy outcomes (Borini et al., 2006; Fernández-Gonzalez et al., 2008; Tesarik et al., 2004). In the presence of high proportions of spermatozoa with DNA fragmentation (>20 %) the risk of infertility increases even if the basic semen parameters were normal. However, if one of the semen parameters was abnormal, the risk of infertility increases already when DNA fragmentation levels are also above 10 % (Giwerzman et al., 2010).

During the late stages of spermatogenesis, the majority of histones are replaced by transition proteins, which are subsequently replaced by more basic and much smaller protein named protamines. The chromatin is twisted around its axis to form unique super coiled structures known as toroids (González-Marín et al., 2012). These structures represent the fundamental packaging units of sperm chromatin in mammals (Agarwal et al., 2012). During sperm transit throughout the epididymis, the toroids become cross-linked and further compacted by disulphide bonds. All the 23 chromosomes are clustered together to form the chromocenter, which is a small and highly compacted mass positioned in the nuclear interior (Zalensky et al., 1995). Nuclear compaction is essential for the protection of the sperm DNA against different exogenous assaults (González-Marín et al., 2012).

Compared with other species, human spermatozoa are characterized by relatively higher basal levels of DNA fragmentation, not only in infertile but also in potentially fertile men (Sakkas et al., 2010). The extent of chromatin compaction in human spermatozoa has been found to be different from other

mammals, apparently due to the variability in the basic protein structures. 15 % of human sperm nuclear proteins are packed by histone, whereas 85 % is protamines bound. This ratio is exceptionally different in many other species where protamines form up to 95 % of the sperm chromatin packaging (Bellve et al., 1988). Histone is known to be much less basic than protamine, thus histone-bound DNA sequences are less tightly compacted and more prone to damage (Agarwal et al., 2012).

Although the exact mechanism is not completely understood, DNA damage in human sperm seems to be a consequence of a defective chromatin condensation and/or an impaired DNA repair mechanism during late spermatogenesis (Agarwal et al., 2012). The chromatin remodelling process is characterized by the elimination of unconstrained DNA supercoils, a process deemed to be essential for the histone replacement by protamine and the transient relief of torsional stress (Marcon and Boissonneault, 2004). This process necessitates the formation of DNA single or double strand breaks by endogenous nuclease activity (Boissonneault, 2002). These DNA breaks, if not properly repaired at an early stage, will develop into DNA fragmentation or genetic mutations on ejaculated sperm (González-Marín et al., 2012; Marcon and Boissonneault, 2004).

A number of methods are currently available for the assessment of sperm DNA fragmentation. Some of these assays can quantify DNA damage directly by the enzymatic labelling of DNA breaks with fluorescent probes (Shamsi et al., 2011). Among these assays are the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay [TUNEL] (Huang et al., 2005), the single cell gel electrophoresis or Comet assay (Sheikh et al., 2008) and in situ nick translation [ISNT] (Gorczyca et al., 1993). Other assays, e.g. the sperm chromatin structure assay [SCSA] (Evenson et al., 2005) and the sperm chromatin dispersion test [SCD], are based on the susceptibility of sperm DNA to acid induced denaturation at the site of damage (Shamsi et al., 2011).

Sperm DNA integrity is essential for normal fertilization and transmission of paternal genetic information to the offspring (Evgeni et al., 2014). DNA fragmentation is a valuable parameter not only for evaluating the functional competence of spermatozoa, but also for the prediction of the rate of fertilization, implantation and embryo development (Fernández-Gonzalez et al., 2008). Increased

proportions of sperm DNA fragmentation have been suggested as a possible risk factor for poor embryo quality and spontaneous miscarriage (Borini et al., 2006; Tesarik et al., 2004). In men with DNA fragmentation rates of < 40 %, the probability of natural conception was 10 times greater than those with DNA fragmentation levels exceeding 40 % (Spanò et al., 2000). Furthermore, the chance of fertilization in IUI was found to be close to zero in men with sperm DNA fragmentation rates higher than 30 % (Evenson et al., 1999; Spanò et al., 2000). Likewise, the probability of achieving pregnancy and delivery following IVF and ICSI was dramatically decreased when the rates of DNA damage exceeded 27 % (Bungum et al., 2004).

2.3.12 Reactive oxygen species (ROS)

The most stable form of an atom is described as a ground state wherein every electron in the outermost shell is paired with a complimentary electron spinning in the opposite direction. In this condition the atom reaches the lowest possible energy levels, it is thus unable to react with surroundings (Agarwal et al., 2008; Du Plessis et al., 2015). A free radical, by contrast, is generally referred to any species or molecule that has one or more unpaired electrons in its outermost shell. This electron imbalance makes the radical unstable and readily reacts with proximate biomolecules (Halliwell and Cross, 1994).

Free radicals comprise a large number of molecules categorized broadly into two distinct types. The first category includes oxygen-derived free radicals, known as ROS e.g. superoxide ($O_2^{\cdot-}$), hydroxyl (OH^{\cdot}), hydrogen peroxide (H_2O_2) and hypochlorite radical (OCl^{\cdot}). The second category includes nitrogen-derived free radicals known as reactive nitrogen species (RNS) e.g. nitric oxide (NO^{\cdot}), nitrogen dioxide (NO_2^{\cdot}). These RNS are sometimes described as a subclass of ROS (Du Plessis et al., 2015).

2.3.12.1 Formation of ROS

Diatomic oxygen (O_2) contains two unpaired electrons in its outermost shell, but is relatively inert as these electrons have parallel spins (Ford, 2004). The $O_2^{\cdot-}$ is a highly reactive oxygen metabolite

produced due to the univalent reduction of oxygen molecules during cell respiration (Koppers et al., 2008). When present at high concentrations, $O_2^{\cdot-}$ is capable of interfering with cellular functions and producing secondary ROS (Agarwal et al., 2008). Further reduction of the $O_2^{\cdot-}$ results in the production of peroxide (O_2^{2-}), which is not a free radical (Ford, 2004). The majority of H_2O_2 is generated as a result of the dismutation of $O_2^{\cdot-}$, which occurs either spontaneously or the enzymatically by SOD (Halliwell and Cross, 1994). H_2O_2 may also be produced directly by numerous enzymes involved in a peroxisomal pathway including, glycolate and monoamine oxidases. The membrane permeable H_2O_2 is poorly reactive, although it can readily be transformed into an enormously reactive OH^{\cdot} via Fenton and Haber Weiss reaction (Halliwell et al., 2000). This reaction necessitates the presence of trace amount of transition metal ions, particularly iron, which is reduced by $O_2^{\cdot-}$ from ferric to ferrous state. The resultant ferrous ions act as catalyst that subsequently increases the rate of conversion H_2O_2 to OH^{\cdot} (Das et al., 2015, Koppenol, 2001).

NO^{\cdot} is primarily generated from L-arginine by the enzyme NO Synthase, with molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) acting as co-substrates. NO^{\cdot} may also be produced by a series of metabolic reactions in human body including pentose phosphate pathway via glucose-6-phosphate dehydrogenase as well as the NADPH (Bolaños et al., 2008). The reaction between NO^{\cdot} and $O_2^{\cdot-}$ results in the generation of peroxynitrite ($ONOO^-$), which is more potent oxidant (Pacher et al., 2007). Various other free radicals have been identified including; ozone, organic peroxy and alkoxy radicals, but have no biological relevance (Du Plessis et al., 2015).

2.3.12.2 Origins of ROS in semen

ROS in seminal plasma can be generated from a wide variety of sources that be either endogenous or exogenous. The following section will focus on the endogenous sources of ROS.

Leukocytes are commonly present throughout the male reproductive tract and can be found in normal semen samples from fertile men. However, If the concentration of the leukocyte exceeds a level of 1×10^6 per mL of semen, leukocytospermia is present (Cooper et al., 2010). A clear correlation has

been demonstrated between the leukocyte concentration and ROS levels in semen (Aitken et al., 1992). Polymorphonuclear neutrophils, about 50–60 % of all seminal leukocytes, represent a major source of ROS in semen. The activation of these leukocytes results in several-fold increases in ROS production (Plante et al., 1994). Peroxidase positive leukocytes; triggered by various factors including infections and inflammations, produce higher levels of reactive free radicals through activation of the membrane bound enzyme NADPH oxidase. This enzyme catalyses the formation of $O_2^{\cdot-}$ via the hexose monophosphate shunt, which represents an alternative pathway of glycolysis results in the generation of a considerable amount of the cytoplasmic NADPH (Babior, 1999; Kessopoulou et al., 1992).

The exact mechanism by which leukocytospermia is implicated in excess of ROS production by spermatozoa is poorly understood. However, such phenomenon might be attributable to direct sperm–leukocyte contact or the potential effect of soluble factors released by the activated leukocytes (Saleh et al., 2002a).

Sperm cells with excess residual cytoplasm are identified as immature and functionally defective (Rengan et al., 2012). Incomplete cytoplasmic extrusion during spermiogenesis occurs concomitantly with trapping an excess amount of enzymes within the cell e.g. glucose-6-phosphate dehydrogenase (G6PD) and NADPH oxidase (Kothari et al., 2010). The presence of an excess in G6PD results in the formation of increased amounts of NADPH via the hexose monophosphate shunt. The intermediate NADPH represents a principal source of electrons for the reduction of oxygen molecules to $O_2^{\cdot-}$, mediated by NADPH oxidase (Du Plessis et al., 2015; Ford 2004). In support of this, higher levels of ROS were observed in the immature spermatozoa isolated by density gradient centrifugation (Gil-Guzman et al., 2001).

Normal spermatozoa themselves may also have the capacity to generate endogenous ROS possibly as a metabolic by-product of aerobic metabolism (Du Plessis et al., 2015). ROS generation by normal spermatozoa is thought to occur through the Krebs's cycle, a cascade of events that eventually lead to the generation of ATP. This process is mediated by a range of cytoplasmic and mitochondrial

enzymes, primarily NADH dependent oxidoreductase [diaphorase] (Gavella and Lipovac, 1992). This enzyme is characterized by its ability to catalyze substrate reduction via transferring electrons from NADH resulting in a reduced substrate and NAD^+ , which is in turn reduced to NADH. The latter acts as a reducing agent to donate electrons to the subsequent pathway in the electron transport chain (Du Plessis et al., 2015; Koppers et al., 2008).

In addition to the endogenous sources, numerous external factors have been shown to contribute to the generation of pathological amounts of ROS in semen. These factors include a growing list of lifestyles and environmental exposures e.g. cigarette smoking (Saleh et al., 2002b), alcohol consumption (Das and Vasudevan, 2007), environmental pollutants like phthalate (Kasahara et al., 2002), as well as various food preservatives (Acharya et al., 2003). Elevated levels of seminal ROS have also been reported in a number of pathological conditions such as spinal cord injury (Padron et al., 1997) and varicocele (Allamaneni et al., 2004).

2.3.12.3 Physiological roles of ROS

Sperm maturation: Normal physiological levels of ROS are crucial for maintaining various vital sperm functions at different transformational stages in the male genital tract. ROS also act as important mediators for proper signal transduction involved in sperm capacitation, hyperactivation and acrosome reaction (Aitken et al., 2004; Edith et al., 2016; Sabeur and Ball, 2007).

Sperm maturation, during spermatogenesis and epididymal transit, occurs through a sequence of events including membrane and nuclear remodelling (González-Marín et al., 2012). Appropriate amounts of ROS appear to be essentially required for the oxidation of cystein-thiol groups of protamines to disulphate bonds, which are necessary for chromatin compaction and stabilization (Aitken et al., 2004). In fact, substantial reductions in ROS generation in sperm and seminal plasma after long-term antioxidant supplementation resulted in a significant increase in chromatin decondensation. The incidence of a high degree of sperm chromatin decondensation may induce cytoplasmic fragmentation in the embryo (Ménézo et al., 2007).

During spermatid differentiation, ROS have shown to be involved in the formation of the mitochondria capsule throughout the oxidation of phospholipid hydroperoxide glutathione peroxidase by H_2O_2 . This enzyme is ultimately converted to a structural protein comprising more than half of the mitochondrial sheath of the mid-piece of the mature sperm (Baker and Aitken, 2004; Puglisi et al., 2005; Roveri et al., 2001).

Capacitation is a multi-step maturational process that takes place in the female genital tract in order to facilitate sperm-oocyte recognition and binding (Chang, 1984). ROS have been described to act as intracellular signalling molecules mediating membrane destabilization, hyperpolarisation and lipid redistribution during the capacitation process (O'Flaherty et al., 2006).

Sperm capacitation remains poorly understood process, which is thought to be initiated by the influx of Ca^{2+} and bicarbonate (HCO_3^-) probably due to the inhibition of plasma membrane Ca^{2+} ATPase as well as the alkalization of the cytosol (Baldi et al., 1996; Du Plessis et al., 2015). In presence of high intracellular levels of Ca^{2+} , ROS particularly $\text{O}_2^{\cdot-}$ triggers the activation of adenylate cyclase, resulting in the formation of cAMP. At elevated concentrations, cAMP induces the activation of protein kinase A (PKA), an enzyme that can modify a neighbouring protein with a phosphate group (Du Plessis et al., 2015). PKA subsequently induces tyrosine phosphorylation of various substrates present primarily in the apical piece of the cell membrane as well as the midpiece and the principal piece of the flagellum (Aitken, 1997; Aitken et al., 2007). Tyrosine phosphorylation during sperm capacitation and related events is tightly regulated most likely via cross-talk and interconnectivity between several biochemical pathways (Naz and Rajesh, 2004). These include the cAMP/PKA pathway and the extracellular signal-regulated kinase pathway an intracellular cascade of signalling proteins from a receptor on the cell surface to the nuclear DNA (Hindley and Kolch, 2002; Naz and Rajesh, 2004; O'Flaherty et al., 2006).

This array of molecular changes results ultimately in significant alterations in the membrane characteristics including; sperm surface protein distribution, membrane fluidity and ionic

permeability in addition to alterations in enzymatic activities and modulation of intracellular constituents (Baldi et al., 1996).

Hyperactivation, as previously mentioned, is a high-energy pattern of sperm motility observed at the site and time of fertilization as a late event of sperm capacitation. In the hyperactivated state, the vigorous whiplash-like beating pattern of the flagellum provides the sperm with propulsion necessary for the penetration of the cumulus cell layer surrounding the egg (Katz et al., 1978; Demott and Suarez, 1992).

Although the molecular mechanism of hyperactivation is poorly understood, a sustained generation of mild $O_2^{\cdot-}$ seems to be essential not only to initiate, but also to maintain hyperactivation. In support of this view, the addition of SOD to already hyperactivated spermatozoa reversed the hyperactivation (De Lamirande and Cagnon, 1993). The beneficial effect of ROS on sperm hyperactivation may not be limited to $O_2^{\cdot-}$. Supplementation of the medium with specific concentrations of H_2O_2 has also been shown to induce sperm hyperactivation (by 37 %) compared to the control (Griveau et al., 1994). This suggests that both $O_2^{\cdot-}$ and H_2O_2 play a role in promoting sperm hyperactivation (De Lamirande et al., 1997).

Acrosome reaction, as mentioned above, is an irreversible exocytotic process that occurs in the final stage of sperm maturation immediately prior to the process of fertilization (Breitbart et al., 1997). *In vivo*, the binding of the sperm to the zona pellucida triggers the initiation of a cascade of molecular signal transduction events, which ultimately results in an increase in intracellular Ca^{2+} levels required for acrosomal exocytosis (Anifandis et al., 2014). As previously described, $O_2^{\cdot-}$ generated via the membrane-bound NADPH oxidase may dismutate into H_2O_2 . Both of these oxygen species, at normal levels, may trigger the acrosome reaction via stimulation of tyrosine phosphorylation of specific proteins on the plasma membrane of sperm head (Du Plessis et al., 2017). The biochemical cascade of the acrosome reaction seems to have common characteristics with those of capacitation including the phosphorylation of the same set of proteins, entry of extracellular Ca^{2+} into the cytosol and the

activation of adenylate cyclase and cAMP (Kothari et al., 2010; Liguori et al., 2004; Naz and Rajesh, 2004).

The presence of ROS within physiological levels also brings about beneficial effects on sperm chemotactic response toward the follicular fluid (Sánchez et al., 2010) as well as the fusion with the female gamete (Aitken et al., 1995). However, an unbalance of the generation and degradation of ROS causes impairment in sperm quality attributable to oxidative damage of the cell (Griveau et al., 1997; Sánchez et al., 2010).

2.3.12.4 Pathological effects of ROS on spermatozoa

It has been estimated that 40 to 88 % of semen samples of non-selected infertile men have increased levels of ROS (Lewis et al., 1995). Supra-physiological levels of ROS have also been reported in nearly 11 % of infertile men with normal semen analysis (Pasqualotto et al., 2001). Furthermore, a strong correlation has been suggested between the seminal ROS levels and pregnancy rates in subfertile couples undergoing IVF (Hammadeh et al., 2006). The pathological levels of ROS in semen may vary significantly among infertile patients (Iwasaki and Gagnon, 1992), and are more likely to be attributed to an excessive ROS formation rather than impaired antioxidant capacity (Zini et al., 1993).

At pathological levels, ROS become highly reactive, causing substantial damage to various types of cellular biomolecules such as nucleic acids, proteins and lipids (Sharma et al., 2012). Several factors have been shown to determine the extent of the oxidative damage ROS may cause. These factors include the concentrations and the type of oxidant, period of exposure, antioxidant capacity, surrounding temperature and oxygen tension (Kothari et al., 2010).

Spermatozoa are very sensitive to ROS damage by lipid peroxidation because their membranes are highly rich in polyunsaturated fatty acids (PUFA), primarily docosahexaenoic acids. These unsaturated fatty acids are primary substrates for peroxidation as they contain methylene group with extremely reactive hydrogen atoms (Oborna et al., 2009). Lipid peroxidation is a chain reaction

initiated when ROS, especially OH^\bullet and Hydroperoxyl (HO_2) generated from $\text{O}_2^{\bullet-}$, combines with a hydrogen atom from a fatty acid to yield a lipid radical. These are unstable radicals reacting quickly with oxygen molecules to form peroxy fatty acid radicals, which are then converted into lipid peroxides. In presence of a transitional metal ion, lipid peroxide is catalysed into OH^\bullet (Ayala et al., 2014). These radicals are capable of abstracting electrons from other PUFA to generate new radicals and thereby propagating the lipid peroxidation chain reaction (Kothari et al., 2010). This chain is ceased when the radicals react with each other to create a non-reactive product called malondialdehyde (MDA). This by-product is widely used as a biomedical marker to estimate the extent of peroxidation damage to spermatozoa (Collodel et al., 2015). Termination of lipid peroxidation chain may also arise in presence of suitable antioxidants (Kefer et al., 2009).

As lipid peroxidation reaction chain sustained membrane fatty acids are progressively lost, resulting accordingly in a decrease in the membrane fluidity, an increase in its non-specific permeability to ions, and inhibition of its bound receptors and enzymes (Lenzi et al., 2004).

In addition to its mediating role in lipid peroxidation, ROS especially NO^\bullet , $\text{O}_2^{\bullet-}$ and OH^\bullet (Epe et al., 1996) may also react with DNA nucleotides producing base modifications and DNA strand breaks (Zribi et al., 2011). Sperm DNA is especially susceptible to oxidative damage, possibly due to the genetic structure of its Y chromosome that cannot repair double strand breaks (Cocuzza et al., 2007). Such susceptibility is further exacerbated by poor chromatin compaction and incomplete protamination (Iommiello et al., 2015). A strong positive correlation has been reported between ROS levels and the proportions of sperm DNA fragmentation (Khodair et al., 2013; Mayorga et al., 2013). Interestingly, single strand breaks seem to be a result of a direct oxidative attack by ROS, whereas double strand breaks are more likely attributable to exposure to lipid peroxidation breakdown products (Badouard et al., 2008). ROS induced DNA damage is characterized by various structural changes in DNA including; cross-linking, base modifications and deletions, strand breaks, chromosomal breakage and/or rearrangement mutations as well as other genotoxic effects (Abdel Haliem et al., 2013; Gill et al., 2010).

ROS has also been implicated to act as an apoptotic stimulus that triggers the mitochondria to release some signalling molecules essential for the activation of programmed cell death (Agarwal et al., 2014b; Mahfouz et al., 2010). Mature spermatozoa from ROS-positive infertile patients showed substantially elevated levels of apoptosis compared with the control group (Agarwal and Said, 2003). However, antioxidant therapy has recently shown to reduce the apoptotic response to oxidative stress (Yüce et al., 2013).

2.3.13 Antioxidants

Under normal conditions, the maintaining of a mild oxidative stress is crucial for the physiological function of intracellular signalling pathways. Redox homeostasis is tightly regulated by an array of antioxidants acting as effective free radical scavengers, which protect spermatozoa against oxidative damage (Trachootham et al., 2008). Antioxidants are “compounds or enzymes that scavenge and inhibit the formation of ROS, or oppose their actions” (Sikka et al., 1995). They are basically classified based on their nature into enzymatic antioxidants and nonenzymatic antioxidants. Enzymatic antioxidants include a group of cellular detoxifying enzymes with powerful antioxidant properties such as, Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (GPx). Non-enzymatic antioxidants include a variety of low-molecular compounds such as; vitamin A, C, and E, ascorbate, carotenoids, glutathione, pyruvate, albumin, uric acid, taurine and hypotaurine. These compounds may act directly as ROS scavengers or indirectly by activating some enzymatic oxidants (Agarwal and Prabakaran, 2005). Spermatozoa have limited intracellular enzymatic defence against oxidative stress, partly due to the cytoplasmic extrusion during spermatogenesis. This deficient capacity is compensated for quite effectively by the antioxidant system provided by the seminal plasma (Grunewald and Paasch, 2012).

2.3.13.1 Enzymatic antioxidants

Superoxide dismutase (SOD) consists a family of “metal-containing enzymes that catalyze the conversion of two $O_2^{\cdot -}$ anions into O_2 and H_2O_2 ”, providing the sperm with significant protection

against peroxidative damage (Alvarez et al., 1987). According to the metal ion at their active site, three SOD isoenzymes have been identified in various components of a mammalian cell. These isoenzymes include the mitochondrial manganese SOD (Mn-SOD), the secretory tetrameric extracellular SOD (EC-SOD) and the dimeric copper-zinc SOD (CuZn-SOD) localized in the cytosol and the intramembranous space (Alvarez and Storey, 1989; Peeker et al., 1997).

The CuZn-SOD is the major SOD isoenzyme in seminal plasma, contributing to about 75 % of its enzymatic activity (Peeker et al., 1997) that compensates for the deficient SOD activity in spermatozoa (O'Flaherty, 2014). The addition of exogenous SOD to sperm suspensions could protect against the oxidative damage and improve sperm motility as well as membrane integrity (Berlinguer et al., 2003; Cocchia et al., 2011; Kobayashi et al., 1991). SOD is also suggested to play a critical role in preventing the early development of ROS induced premature capacitation and inopportune hyperactivation (De Lamirande and Gagnon, 1995). In addition, a positive correlation was reported between semen SOD activity and various sperm parameters including sperm concentration, total motility, morphology and viability (Hsieh et al., 2002; Murawski et al., 2007; Shamsi et al., 2010).

Catalase (CAT) is a common antioxidant enzyme found mainly in peroxisomes. It catalyses the decomposition of H_2O_2 to O_2 and H_2O . CAT activity in human spermatozoa is limited, but it is detectable in seminal plasma, contributed mainly by prostatic secretions (Jeulin et al., 1989; Sikka et al., 1995). Protein expression of CAT in seminal plasma was positively correlated with sperm progressive motility and morphology (Macanovic et al., 2015). In asthenozoospermic patients, a significantly lower semen CAT activity was reported when compared with normozoospermic men (Jeulin et al., 1989).

Glutathione peroxidase (GPx) is another key enzymatic antioxidant protecting spermatozoa against ROS-mediated oxidative attack. GPx comprises a family of enzymes with different characteristics found in various reproductive tissues. They catalyse the reduction of H_2O_2 and a range of lipid hydroperoxides to H_2O and alcohol respectively, using reduced glutathione (Sies et al., 1997). The most abundant GPx isoenzymes are GPx1, which is expressed in spermatozoa and the genital tract

and GPx4, expressed primarily in the testicular tissues (Tvrdá et al., 2011). Activities of these enzymes in seminal plasma have been linked to sperm quality in fertile and infertile men (Foresta et al., 2002; Idriss et al., 2008).

2.3.13.2 Non-enzymatic antioxidants

Vitamin E is a major chain-breaking antioxidant that protects the sperm membranes against peroxidation by trapping and scavenging $O_2^{\cdot-}$, H_2O_2 and OH^{\cdot} in a dose-dependent manner (Hull et al., 2000; Suleiman et al., 1996). Vitamin E supplementation led to enhanced enzymatic antioxidant defence and decreased levels of MDA, a biomarker of lipid peroxidation of the sperm membranes (Suleiman et al., 1996). Furthermore, the addition of vitamin E to the cryoprotective media resulted in a significant improvement in post-thaw motility and DNA integrity (Kalthur et al., 2001).

Vitamin C is another chain-breaking antioxidant found primarily in seminal plasma, representing up to 65 % of its antioxidant capacity (Agarwal and Prabakaran, 2005). In addition to its essential role in protecting the sperm from oxidative damage, vitamin C also prevents sperm agglutination and assists in recycling the oxidised vitamin E (Angulo et al., 2011). Several studies have established the dose-dependent beneficial effects of vitamin C on different sperm quality parameters such as motility (Abel et al., 1982) and morphology (Colagar and Marzony, 2009). Dietary supplementation with vitamin C has also shown to ameliorate oxidative stress caused by cigarette smoke (Fraga et al., 1991).

Glutathione is one of the most abundant endogenous non-thiol proteins in mammalian cells (Irvine, 1996). Its sulfhydryl group acts as a proton donor and is responsible for its antioxidant properties. Oxidation of the sulfhydryl group results in the formation of glutathione disulphide, which is reduced instantly by glutathione reductase to regenerate glutathione (Zhao et al., 2011). A significantly lower glutathione content of spermatozoa were reported in infertile men compared with that of fertile controls (Ochseedorf et al., 1998). Furthermore, the utilization of glutathione therapy in certain andrological pathologies resulted in a substantial improvement in both sperm parameters and cell membrane integrity (Lenzi et al., 1994).

Coenzyme Q-10 is a lipid soluble antioxidant related to low-density lipoproteins and found primarily in the sperm midpiece. It traps free radicals and protects the sperm against peroxidative damage (Frei et al., 1990). It may also be implicated in recycling vitamin E and inhibiting its pro-oxidant capacity (Karbownik et al., 2001). As a component of the mitochondrial respiratory chain, the Coenzyme Q-10 can assist in promoting energy production to enhance sperm motility (Choudhary et al., 2010; Lewin and Lavon., 1997).

Several other molecules with potential antioxidant activities have been reported in the reproductive system e.g. uric acid (Guz et al., 2013), bilirubin (Sedlak et al., 2009) and albumin (Roche et al., 2008). These endogenous substances might not react with oxidants directly, but may indirectly contribute to the protection of sperm oxidative homeostasis.

2.4 Computer-aided sperm analysis (CASA)

CASA is an automated system with hardware and software packages designed to visualize and digitalize a series of sequential images of the sperm, therefore providing accurate, precise and objective information about different aspects of sperm quality parameters, namely sperm concentration, motility, morphology and morphometry (Lu et al., 2014; Talarczyk-Desole et al., 2017). The basic principle for CASA is that the various sperm images from the microscope field are captured by the camera and converted into digital images. The captured microscopic field is visualized using a dark field or a negative-high-phase contrast image, in order to show the sperm heads as clear white images against a black background. The core of the sperm head remains consistently bright even when the sperm moves, as the sperm head displacement does not influence the intensity of its white image. The captured images of the sperm head are translated to digital data. Subsequently, the computer analyses the trajectory of each individual sperm according to the predefined frame rate and the range of pixels of the sperm head (Holt et al., 2007; Mortimer, 2000).

In the mid-1980s, the earliest commercial CASA instrument was introduced to the market as a method for tracking sperm motion. Unfortunately, these early generation machines did not show sufficient

accuracy for practical applications due to fundamental limitations, including the lack of standardizing criteria (Mortimer et al., 2015). In an attempt to standardize the protocols and applications of CASA for the assessment of human semen, the ESHRE Andrology Special Interest Group convened three international consensus meetings (Mortimer, 1995; 1996; Mortimer et al., 1998), during which several limitations of CASA were addressed and recommendations were formulated. However, during the 20 years that have passed since the publication of the most recent ESHRE guidelines, many of the problems that constrained earlier systems have been addressed as major innovations in bioengineering and computer technology have been made. The massive improvement in CASA technology in the last three decades was accompanied by a substantial increase in the number of scientific articles, which employed CASA in both human and animal sperm studies (Figure 2.6). The successful application of CASA in research has encouraged its incorporation into routine clinical practice, as more than half of the andrology laboratories in China, for instance, have been reported to use CASA systems (Lu et al., 2010).

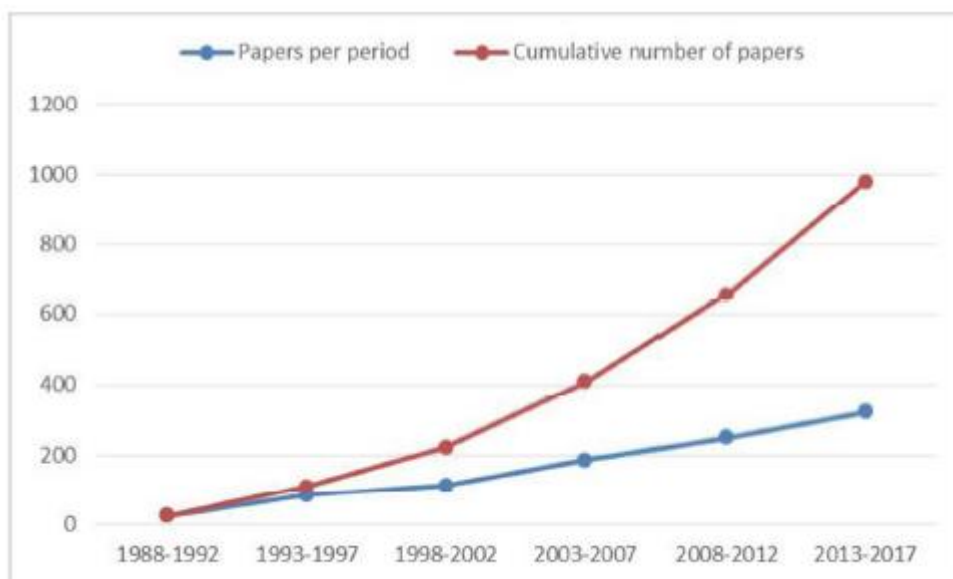


Figure 2. 6: Increased CASA-related publications for each five-years between 1988-2017. Adapted from (Van der Horst, et al., in press).

2.4.1 Advantages of CASA

2.4.1.1 Sperm motility

The assessment of sperm motility is a fundamental part of semen analysis as it provides essential information about the functional competence and the fertilizing potential of spermatozoa (Gunalp et al., 2001; Simon and Lewis, 2011). The manual microscopic estimation of sperm motility is subjective and highly associated with inter- and intra-laboratory variations. Within the same semen sample, variations of 30–60% have been reported in manually assessed human and animal ejaculates (Verstegen et al., 2002). In contrast to this subjective method, CASA is a powerful tool that has the capacity for the objective analysis of motility in larger populations of sperm with increased accuracy (Fréour et al., 2010). CASA-derived motility parameters include total motility, progressive motility, non-progressive motility, static, fast progressive [type a], slow progressive [type b], non-progressive [type c] and immotile [type d]). Furthermore, CASA systems allows for obtaining verifiable data, since previously analysed video images can be re-evaluated for periodic internal and external quality control assessments.

2.4.1.2 Sperm kinematics

In addition to being powerful analytical tools for assessing sperm motility, CASA systems provides additional details on sperm motion via determining their kinematic characteristics. Each individual sperm in the field of view is identified and a series of digital images of the spermatozoal head movement is captured. This allows for the reconstruction of their individual trajectories (Mortimer et al., 2015). CASA-based kinematics include the following measurements, VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF. These kinematic parameters provide valuable information on sperm quality, which cannot be obtained by the subjective evaluation using manual microscopic examination, and could become important components of male fertility evaluation, thereby enhancing the routine semen analysis (Estofan et al., 2017; Ranganathan et al., 2001; Soler et al., 2017).

2.4.1.3 Sperm hyperactivation

Hyperactivation is a form of non-progressive motility observed at the site and time of fertilization and is characterized by extremely asymmetrical flagellar undulations with higher amplitudes and lower frequencies leading to highly curved or whiplash swimming trajectories (Demott and Suarez, 1992; Katz et al., 1978). Hyperactivated motility of spermatozoa was initially evaluated manually by describing the flagellar movement patterns subjectively based on visual observations. However, due to the fact that sperm hyperactivation is flagellar phenomenon associated with changes in the amplitudes and the frequencies of the flagellar waves, the manual analysis of sperm hyperactivation remains extensively laborious, time consuming and uncontrolled (Suarez, 2008). Recently, quantification of the hyperactivated population of spermatozoa has been enabled and simplified since visual analysis has been superseded by automated measuring procedures. CASA systems have been strongly recommended to be a more practical option for the identification of hyperactivated spermatozoa (Mortimer, 2000; Mortimer et al., 2015). The identification of hyperactivated sperm populations by CASA is performed by employing a combination of sperm kinematic parameters including VSL, LIN and ALH. Accordingly, hyperactivated motility for human spermatozoa is represented as a Boolean argument, which is defined by the following kinematic values $VCL > 150 \mu\text{m/s}$, $LIN < 50 \%$ and $ALH > 7.0 \mu\text{m}$ when analysing at 60 images per second. In contrast, non-hyperactivated or progressively motile spermatozoa are characterized by straight trajectories with relatively lower VCL, relatively higher VSL and particularly lower ALH values (Mortimer et al., 2015).

2.4.1.4 Sperm morphology

Despite the seeming simplicity of spermatozoa, these specialized cells are highly differentiated and evolved in shape and size. The possible link between sperm morphology and fertility has already been recognized since the middle of the previous century (Macleod and Gold 1951). However, the predictive value of sperm morphology in male fertility has always been a controversial issue; mainly due to the wide variability of manual morphology evaluation within and between laboratories, which

subsequently limited its practical application (Eustache and Auger, 2003; Maree et al., 2010; Van der Horst and Du Plessis, 2017). As reviewed by Yániz *et al.* (2015), quality control programs for the manual microscopic analysis of the human ejaculate have reported within-technician coefficients of variation of between 10-80%. The analysis errors associated with the subjective visual assessment of sperm morphology makes it even more difficult to interpret the obtained results, which can subsequently lead to inappropriate treatment plans.

For the objective assessment of sperm morphology, the automated sperm morphology analysis (ASMA), generally referred to as computer-aided sperm morphology analysis (CASMA), software was developed and first appeared on the market in the early 1990s (Kruger et al., 1993). This technology assists considerably in eliminating the human evaluation biases through providing faster, more accurate and quantifiable morphometric measurements (Garrett et al., 2007).

2.4.1.5 Sperm morphometry

Apart from being a reliable tool for the objective assessment of the overall proportion of sperm morphology in the ejaculate, CASA instruments also allows for the direct measurement of individual sperm dimensions. Subsequently, the following novel sperm head and midpiece morphometric parameters have been identified, Head Length (μm), Head Width (μm), Head Area (μm^2), Head Perimeter (μm), Chord (μm), Head Angle (degrees), Head Linearity (%), Head Roughness, Head Regularity, Head Ellipticity, Acrosome cover (%), Midpiece Width (μm) and Midpiece Angle (degrees). The latest generations of CASA systems incorporate the minimum and maximum cut-off points for each of these morphometric variables in order to determine normal sperm morphology in various mammalian species, including humans. Thus, the automatic quantification of species-specific sperm morphometry subpopulations can be established based on clear criteria. Furthermore, the combination of sperm kinematic and morphometric subpopulations not only delineate the distinctive characteristics of the ejaculate, but also assist in elucidating the relationship between sperm structure and function (Soler et al., 2017), which would consequently improve the clinical relevance for semen analysis.

2.4.2 Clinical relevance of CASA measurements

Due to the substantial efforts towards improving the technical performance and efficiency of current CASA systems, it necessitates and has become crucial to evaluate the biological relevance of CASA-derived parameters in predicting male fertility potential. CASA-derived measurements have been shown to be of great value in monitoring the subtle changes in the distribution of spermatozoa amongst different motility and velocity subpopulations in response to various physiological conditions (Elzanaty et al., 2005; Fréour et al., 2012) and environmental exposures (Louis et al., 2014; Mukhopadhyay et al., 2010), which cannot be observed manually by optical microscopic analysis. Studies have shown considerable differences in the CASA kinematics, VCL and LIN, between fertile and infertile men with unexplained fertility (Aitken et al., 1982; Vantman et al., 1989). Similarly, non-hyperactivated spermatozoa from fertile men were characterised by significantly lower values of VAP, VCL and ALH, along with increased LIN and STR as compared to non-hyperactivated spermatozoa from men with unexplained infertility. By contrast, after two hours of incubation in a capacitating medium, hyperactivated spermatozoa from fertile men showed a considerable decrease in VSL and LIN as well as an increase in ALH and BCF when compared to hyperactivated spermatozoa from infertile men. This suggests that spermatozoa from fertile men are likely to be more capable of undergoing hyperactivation as they displayed higher values of ALH and BCF than those of infertile men (Peedicayil et al., 1997).

Several studies have addressed the role of CASA-derived parameters in predicting male infertility. In a study, which included a large number of semen samples collected from men from the general population, the proportion of sperm motility, defined as VCL >25 μm , was found to be a dominant factor in predicting the chance of conceiving naturally (Larsen et al., 2000). Similarly, in a large group of subfertile couples, VSL along with the proportion of spermatozoa exhibiting head morphology characteristics similar to those spermatozoa that have been found to typically bind to the zona pellucida were significantly and independently associated with increased conception rates (Garrett et al., 2003). In various other studies, fertility rates have also been strongly associated with higher

proportions of spermatozoa having smaller and more elongated heads (Maroto-Morales et al., 2015; Ramón et al., 2013) and displaying fast and linear movements (Ramón et al., 2013) as measured by CASA/CASMA. Furthermore, CASA parameters including, sperm concentration, motility, and rapid velocity have also been reported to be reliable predictors for pregnancy outcome after IUI when combined with superovulation strategies in couples with unexplained infertility. It is therefore clear that many CASA instruments correlate with fertilization and pregnancy. Thus employing CASA would be beneficial in the diagnosis and counselling of patients prior to deciding on proceeding with specific treatment strategies such as IUI, IVF or ICSI.

2.4.3 Limitations of CASA

Despite being a reliable tool that has the capacity to provide detailed measurements of sperm head and midpiece dimensions, most commercially available CASA systems are not capable of analysing the sperm tail characteristics, and consequently limits the application of this technology in the clinical settings (Mortimer et al., 2015). Furthermore, CASA systems are not ready-to-use robots and can be influenced, as any other automated technique, by several artefacts related to inappropriate settings and technical errors (Kraemer et al., 1998; Ibănescu et al., 2016). With recent advances made in CASA software, many of the limitations affecting the CASA performance of measurement have been partially or totally negated. For instance, when assessed manually, sperm motility is defined and categorised based on its flagellar movement and beating pattern, while CASA depends primarily on tracking the displacement of the sperm head. It has been claimed that the assessment of sperm motility percentage using CASA might be unreliable due to the potential misidentification of particulate debris as immotile spermatozoa (WHO, 2010). However, the recently developed SCA (5.4 and 6) CASA models, as used in the current study, are incorporated with intelligent filters eliminating some particles of similar size than sperm, but more importantly utilizing positive phase contrast where most background images are now visualized in black and accordingly not imaged as part of the white reflecting sperm. Furthermore, a feature called “drifting” can be set to eliminate not only Brownian motion, but also minor flow and even help to counteract detecting an immotile sperm that is slightly

displaced as motile because of a collision with a motile sperm . These features allow for more accurate and objective assessment of sperm count and motility (Mortimer et al., 2015).

Before any assessment can be made, standardization and optimization of the operational settings remains essential for all computerized measuring instruments. The various CASA systems and models operate according to similar basic principles. However, the sample handling techniques as well as the characteristics of the algorithms and software for sperm identification and trajectory reconstruction might lead to a source of subjectivity among laboratories (Ibănescu et al., 2016; Mortimer et al., 2015; Rijsselaere et al., 2003). Consequently, inconsistent outcomes may arise when comparing data from studies using the same CASA system, but with different operational settings such as light settings, frame rate, image acquisition frequency, chamber type and depth, velocity and kinematic cut-off points as well as the recognition criteria for sperm head and immotile particles (Gączarzewicz, 2015; Schleh et al., 2013). Nevertheless, potential errors can largely be minimized to permit comparison of data obtained by different systems and technicians through the use of harmonized instrument settings and standardization techniques. In support of this, no major inter-laboratory variations were observed in the automatic analyses of various sperm characteristics when the same semen samples were assessed using different CASA systems, which were operated by independent technicians, but installed with equivalent operational settings (Akashi et al., 2010; Holt et al., 1994; Lu et al., 2014; Maree et al. 2010).

2.4.4 Future of CASA

The successful collaboration and fruitful cooperation between science and industry has resulted in establishing CASA as a reliable tool that has the capacity to quantitatively assess sperm motility, kinematics as well as morphological and morphometric features, in a rapid and precise manner. The combination of these basic characteristics with advanced functional parameters will enhance the diagnostic value of semen analysis and provide a more accurate as well as quantitative approach for the assessment of idiopathic male infertility. Therefore, for the future development of CASA technology, advanced markers of sperm functionality (i.e. mitochondrial function, DNA status,

hyperactivation, cervical mucous penetration) need to be integrated. This will enable a precise objective description of numerous aspects of sperm/seminal quality based on automated assessment.

2.5 Factors affecting sperm quality

In general, semen quality is influenced by a variety of intrinsic and extrinsic factors. Individual variations in semen parameters have been shown to be the result of various factors which can be predictable and controlled for. These factors include abstinence period, ejaculation frequency and collection method. Other factors which have been potentially associated with changes in the chemical and physical semen properties include; the subject's age, general health and lifestyle, infection, inflammation and dysfunction of male sex glands, urogenital surgery as well as several therapeutic and the environmental exposures (Barazani et al., 2014; Du Plessis et al., 2013). The subsequent section will briefly review the main factors that influence semen properties.

2.5.1 Age

Although there is no identified critical threshold for sperm production in men, the weight of evidence indicates that advancing male age is related to substantial reductions in several aspects of semen quality (Kidd et al., 2001). A progressive decrease in both seminal volume and the percentage of sperm motility was reported between the ages of 22 and 80. The average annual decline in the overall sperm motility from 22 years onwards has been estimated to be 4.7 % (Eskenazi et al., 2003). Likewise, a strong trend of decline was reported in semen volume from 1 % for men aged 30 years to 40 % for men aged 50 years (Plas et al., 2000). Aging is also accompanied by an increase in the incidence of abnormal forms of spermatozoa, with coiled tails and microcephalic heads being the most common defects (Schwartz et al., 1983). Age might not only affect conventional semen quality, it may also affect the chromatin integrity of the spermatozoa. In support of this, a strong association was shown between age and DNA fragmentation in men 18–55 years of age (Spano et al., 1998).

The aging process is associated with several cellular and physiological changes, which may induce the deterioration observed in semen quality. These changes include; insufficient accessory glands

secretions, narrowing and sclerosis of the tubular lumen, impaired spermatogenic activity, degeneration of germ cells, and reduced number and function of Leydig cells (Eskenazi et al., 2003, Kidd et al., 2001).

2.5.2 Smoking

The deleterious effects of cigarette smoking on sperm quality have been widely reported (Belcheva et al., 2004; Saleh et al., 2002b; Sepaniak et al., 2006). Nicotine is a major toxic constituent of cigarette smoke and is found at high levels in smokers (Görnig and Schirren, 1996). In male rats, nicotine treatment resulted in a significant decrease in sperm count, motility and normal morphology. The adverse effect of nicotine on sperm parameters was shown to occur in a dose-dependent manner and improved by nicotine cessation (Oyeyipo et al., 2011). In most studies, smokers have been reported to have lower semen volume, sperm count, sperm motility viability and normal morphology compared with non-smokers (Meri et al., 2013; Sharma et al., 2016). Cigarette smoking has also been associated with increased seminal oxidative stress levels (Saleh et al., 2002b), alterations of cell membrane lipids (Belcheva et al., 2004) and increased sperm DNA fragmentation (Sepaniak et al., 2006). It appears that there are no safe levels of cigarette consumption, although the decline in semen quality has been directly proportional to the duration (Zhang et al., 2000) and the number of cigarettes smoked (Gaur et al., 2007).

2.5.3 Alcohol consumption

Alcohol abuse has been associated with progressive deterioration in semen quality (Jensen et al., 2014). A link has been suggested between prenatal alcohol exposure and a number of semen quality parameters including semen volume, sperm concentration and T.S.C. (Ramlau-Hansen et al., 2010). In a case-control study conducted in Japan, alcohol intake was found to be more common in infertile men than in fertile men (Tsujimura et al., 2010). Furthermore, a reduced number of normozoospermic cases was reported amongst alcoholics compared with controls, with the majority of heavy drinkers showing higher percentages of morphologically abnormal spermatozoa (Gaur et al., 2010). A

significant improvement in sperm parameters was observed after three months of alcohol consumption discontinuation (Sermondade et al., 2010). Despite the exclusive evidence on the involvement of alcohol consumption in the increase of systemic levels of oxidative stress (Cederbaum et al., 2009), the specific relationship between sperm oxidative stress and alcohol has yet to be elucidated (Kefer et al., 2009).

2.5.4 Obesity

Several studies have linked the increased body mass index with impaired semen quality (Martini et al., 2010; Wen-hao et al., 2015). Compared with normal weight males, obese males are thought to be three times more likely to have lower WHO semen parameters (Hajshafiha et al., 2013). The prevalence of oligozoospermia was shown to be higher among overweight or obese men (Sermondade et al., 2012). In a recent large population-based cohort study (Belloc et al., 2014), an inverse association was reported between obesity and seminal volume, T.S.C. and sperm concentration, but not with sperm morphology. A significantly higher incidence of DNA fragmentation was also reported in obese men, possibly attributable to induced oxidative stress (Dupont et al., 2013; Kort et al., 2006).

2.5.5 Recreational drugs

The use of illicit drugs appears to be associated with progressive deterioration in semen quality and reproductive system. More than 30 % of the exclusive marijuana smoking men included in the study by Kolodny et al. (1974) were oligozoospermic, possibly due to inhibited testosterone secretions, which ultimately impairs spermatogenesis. Bracken et al. (1990) assessed the effect of cocaine use on sperm concentration, motility and morphology and found that the incidence of sperm concentration lower than $20 \times 10^6/\text{mL}$ was more common in men who have used cocaine more frequently during the last two years. Furthermore, chronic exposure of male rats to ecstasy, a psychoactive drug consumed largely by young population, resulted in higher proportions of spermatozoa with fragmented DNA (Barenys et al., 2009). Similarly, the *in vitro* exposure of human spermatozoa to delta-9-

tetrahydrocannabinol, the primary psychoactive cannabinoid in marijuana, impaired progressive motility, velocity, and acrosome integrity (Whan et al., 2006). Interestingly, delta-9-tetrahydrocannabinol has also been associated with impaired mitochondrial oxygen consumption in human spermatozoa (Badawy et al., 2009).

2.5.6 Ejaculatory abstinence

The WHO criteria for semen analysis provide a standard approach for the prognosis of fertility and diagnosis of infertility in men. These criteria have been universally adopted by most human andrology laboratories during the last three decades (WHO, 1980, 2010). Therefore standardized semen analysis, according to the WHO, remains the initial screening and cornerstone for the evaluation of male fertility.

According to the prescribed guidelines of the WHO, subjects must remain abstinent for a minimum period of 48 hours, but not longer than seven days prior to collecting a sample for a standard semen analysis (WHO, 2010). Whilst, more firmly abstinence intervals of three to four days have also been suggested by the NAFA and ESHRE (Kvist and Björndahl, 2002). However, the basis for this recommendation remains uncertain and contradictory.

Experiments performed to compare the fertilizing capacity of rat spermatozoa recovered from different compartments of the epididymis showed that spermatozoa sampled from the proximal cauda were significantly superior to those from the distal cauda region or vas deferens (Moore and Akhondi, 1996). Similarly, moderate aging of hamster spermatozoa in the cauda epididymis was associated with a decreased fertilizing capacity and an impaired ability of spermatozoa to undergo the acrosome reaction (Cuasnicu and Bedford, 1989). These findings indicate that the typical functional status of caudal spermatozoa cannot be maintained for longer than the average length of time that they usually remain in their epididymal storage before being replaced by younger spermatozoa (Cuasnicu and Bedford, 1989).

During the last half century, various studies have sought to determine the optimal time frame for ejaculatory abstinence and the results are often found to be contradictory. In general, these studies assessed a wide range of abstinence period cut-offs (≤ 1 to 18 days). Shortening the abstinence time to ≤ 2 days has shown to increase pregnancy rates after ovulation induction with IUI (Marshburn et al., 2010). Prolonged sexual abstinence has generally been reported to increase semen volume; sperm concentration and the T.S.C. (Agarwal et al., 2016; Marshburn et al., 2014; Mayorga-Torres et al., 2015; Sunanda et al., 2014). However, the overall quality of spermatozoa is known to be affected by the efficiency of epididymal storage and the rate at which spermatozoa pass from the proximal to the distal cauda region, that in turn vary depending on the frequency of ejaculation (Johnson and Varner, 1988; Turner, 2008). The proportions of progressive motility (Bahadur et al., 2016a; Mayorga-Torres et al., 2015) and total motility (Choavaratana et al., 2014; Valsa et al., 2013) were found to decrease substantially with increasing the abstinence period, whereas no significant associations were noticed in other studies (Jurema et al., 2005; Mayorga-Torres et al., 2015; Sánchez-Martín, et al., 2013). The only study that has investigated the effect of abstinence on the kinematic properties of spermatozoa, as assessed by CASA, was conducted by Elzanaty et al. (2005). This study found an inverse association between the abstinence period and VSL and LIN, whereas VAP and VCL were not significantly different among groups.

There are only few studies available on the impact of abstinence time on the advanced sperm functional parameters such as ROS production and DNA integrity, while their findings are apparently inconsistent. Some studies have revealed that shortening the abstinence time could result in a significant decrease in sperm DNA fragmentation (Agarwal et al., 2016; Sánchez-Martín et al., 2013; Sukprasert et al., 2013) and ROS levels (Mayorga-Torres et al., 2016). Other studies, however, reported insignificant differences (Desai et al., 2010; De Jonge et al., 2004; Mayorga-Torres et al., 2015). In a unique report, Marshburn et al. (2010) showed a significant increase in the total antioxidant capacity with short abstinence period.

Accordingly, the typical time after which semen samples should be collected for standardized analysis remains unclear and needs to be further investigated. In addition, no comprehensive study as of yet investigated the effect of a short abstinence period lasting four hours on sperm motility, viability and morphology, in addition to various existing advanced sperm functional parameters such as acrosome reaction, sperm ROS and DNA fragmentation as well as seminal plasma antioxidant capacity.

Chapter 3: Materials and Methods

The research protocol including the materials used and the subsequent methods that were followed to perform the different measurements throughout the study will be described thoroughly in this chapter.

A brief outline of the experimental protocol in Figure 3.1 illustrates the sequence of analyses.

3.1 Study volunteers

Before the commencement of the study, ethical approval was obtained from the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, Stellenbosch University (Ethics Reference: 15/02/045). Informed written consent was obtained from all donors and the study was performed in accordance with the Declaration of Helsinki (2013). Freshly ejaculated semen samples, with scheduled periods of sexual abstinence, were collected for this study from 100 potentially fertile, healthy males (20 to 30 years of age), taking part in the sperm donor program at the Stellenbosch University Reproductive Research Group (SURRG). Information about the donor's identification and age, date and time of semen collection, abstinence period, the sample's appearance, colour, odour, liquefaction, agglutination as well as any remarkable conditions were recorded.

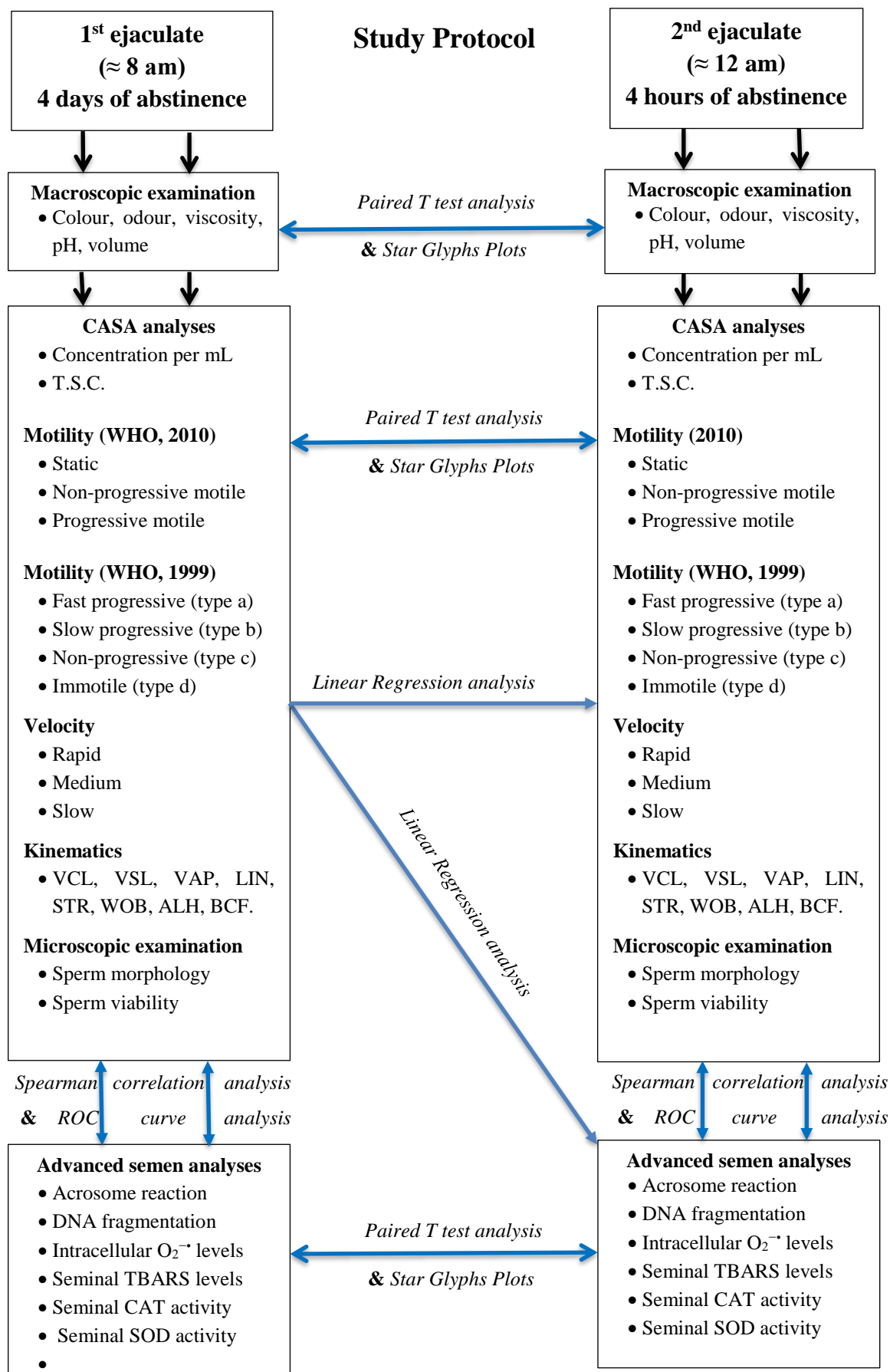


Figure 3. 1: Flow chart showing a simplified experimental protocol

3.2 Basic Semen analysis

3.2.1 Semen sample collection

All samples were collected according to the WHO guidelines (WHO, 2010), in a separate room near to the laboratory, by means of masturbation without lubricant, into a labelled sterile wide mouth plastic container anonymized through alphanumeric coding. The first sample was collected at 8:00 am after a sexual abstinence period of 4 days. The second sample was collected from the same donor after 4 hours subsequent to the first collection. Samples were delivered to the laboratory within an average of 10 minutes and placed immediately in an incubator (Heal Force® Smart Cell CO₂, Nison™, Shanghai, China) and allowed to fully liquefy (37°C, 5 % carbon dioxide [CO₂] 95 % humidity, 30 minutes) and then analysed within 1 hour of collection. One hundred sets of samples were included in this study on the basis of the following inclusion criteria for the first sample: sample volume ≥ 1.5 mL, sperm concentration $\geq 15 \times 10^6$ /mL and total sperm motility ≥ 40 % (WHO, 2010).

3.2.2 Initial macroscopic examination

Subsequent to semen liquefaction at 30 minutes, but no longer than 1 hour after ejaculation, the sample was inspected for colour, appearance, and odour.

3.2.3 Volume

Semen samples were decanted directly from the plastic collection container following the post-ejaculate liquefaction period into a disposable graduated 15 mL plastic Falcon tube and weighed on a micro scale (MonoBloc AB204-S) standardized by a similar empty plastic Falcon tube. The semen volume was recorded in grams and converted to millilitres, considering the density of semen to be 1 g/mL.

3.2.4 The pH

The semen pH was assessed after liquefaction at a uniform time, within 1 hour of ejaculation, by means of pH indicator paper (Merck Millipore, Darmstadt, Germany) with graduated colours indicating pH from 6.4 to 8.0.

3.2.5 Viscosity

Semen viscosity was assessed, according to filling time of a single chamber in a disposable 8 chamber 20- μ m depth slide (SC 20-01-08-B; Leja® Products B. V., Nieuw-Vennep, the Netherlands). The results were quantified according to Rijnders et al. (2007) and expressed in the unit Centipoise (Pc).

$$y = 0.34(x) + 1.34$$

(x = filling time in seconds)

3.2.6 Sperm concentration and motility

The sperm concentration and motility/kinematic parameters were determined with Computer Aided Sperm Analysis [CASA] (Sperm Class Analyzer version 5.4 - SCA®, Microptic, S.L., Barcelona, Spain) with a disposable eight-cell chamber Leja slide (LJ; 20- μ m depth; Leja® Products B. V., Nieuw-Vennep, the Netherlands) at 37°C. The SCA® is equipped with a Basler A312fc digital colour camera (Microptic, S.L., Barcelona, Spain), mounted on a Nikon E200 Microscope (IMP, Cape Town, South Africa) and a stage warmer (Omron™, Kyoto, Japan) which was heated to 37°C. The camera settings for the SCA® system are listed in Table 3.1.

Table 3. 1: Camera settings for the SCA® system

	Parameter	Setting
	Acquisition mode	Timed
Acquisition control	Exposure time	19900
	Time base	20 μ s
	Exposure time	995
	Acquisition frame rate	50 FBS
Counter and timer controls	Time duration Raw	4095
	Gain	435
Analog controls	Black level	168
	Balance ration	77
	Balance ration	1.20313

FBS = frame per second.

In accordance with the manufacturer's guidelines, 2 μ l of the pre-incubated semen (37°C, 5 % CO₂) was loaded into a single chamber of the Leja slide using an Eppendorf micropipette. If bubbles were found, another chamber was loaded. The slide was then allowed to rest on the warm stage (37°C, 30sec) to avoid liquid flow in the chamber. All the preparations were made with pre-warmed (37°C) slides and pipette tips. A minimum of 1000 spermatozoa per semen sample were analysed in several systematically selected areas within the central part of each chamber. The CASA parameters assessed are listed in Table 3.2. As displayed by the SCA® system, various motility colour tracks are presented in Figure 3.2.

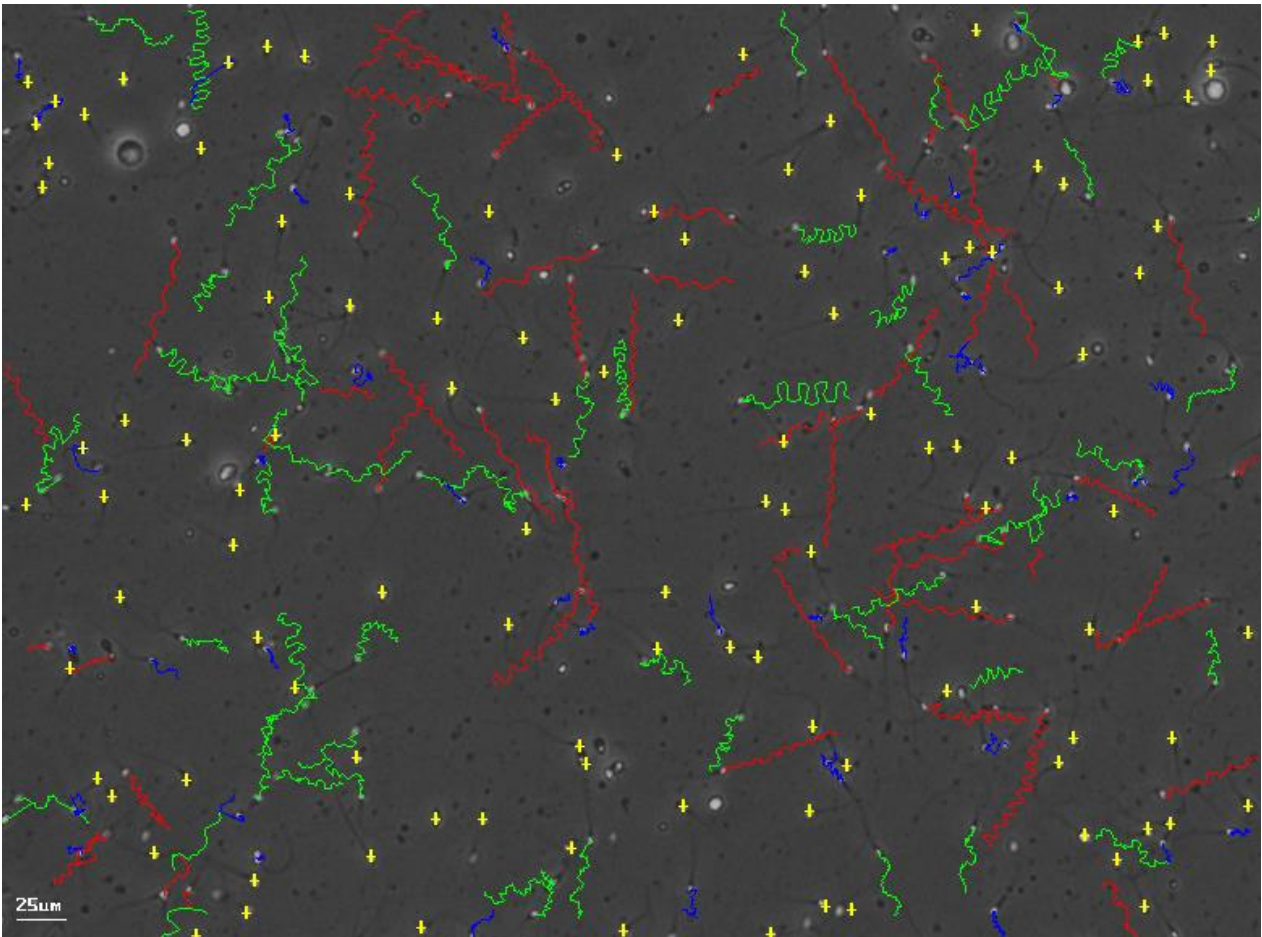


Figure 3. 2: SCA® analysis displaying the different colour paths for motility rating. Red (Type A); green (type B); blue (Type C) and yellow (type D)

The following SCA® settings were used; green filter; Ph1 condenser; positive phase contrast observation setting; brightness ± 400 ; contrast ± 100 ; objective, 10x; eyepiece, 10x; capture, 50 images per second; chamber, Leja 20; scale, 10x; $2\mu^2 < \text{particle area} < 50\mu^2$; VCL, $25\mu\text{/s} < \text{slow} < 40\mu\text{/s}$, $40\mu\text{/s} < \text{medium} < 50\mu\text{/s}$, $50\mu\text{/s} < \text{rapid}$; progressivity, $>80\%$ of STR; circular, $<50\%$ LIN; connectivity, 12; VAP points, $5\mu\text{/s}$; filter, on; temperature, 37°C . WHO reference values adopted in this study are as follows: sperm concentration, $\geq 15 \times 10^6/\text{mL}$; total motility $>40\%$; progressive motility $>32\%$.

Table 3. 2: SCA® concentration, motility and kinematic parameters

Setting	Parameter	Unit
Concentration	Concentration	10 ⁶ /mL
	T.S.C.	10 ⁶ /ejaculate
Motility (WHO, 2010)	Static	%
	Non-progressive motile	%
	Progressive motile	%
Progression (WHO, 1999)	Fast progressive (type a)	%
	Slow progressive (type b)	%
	Non-progressive (type c)	%
	Immotile (type d)	%
Velocity	Rapid	%
	Medium	%
	Slow	%
Kinematics	VCL	µm/s
	VSL	µm/s
	VAP	µm/s
	LIN	%
	STR	%
	WOB	%
	ALH	µm
BCF	Hz	

WHO = World Health Organization, T.S.C. = total sperm count, VCL = Curvilinear velocity, VSL = Straight-line velocity, VAP = Average path velocity, LIN = Linearity, WOB = Wobble, ALH = Amplitude of lateral head displacement, BCF = Beat-cross frequency, µm = micrometre, s = second, Hz = hertz.

3.2.7 Sperm morphology

For sperm morphology assessment, approximately 10 μl of semen ($\geq 20 \times 10^6$ spermatozoa/mL), was placed near one end of a labelled microscope slide (76 x 26mm). A second slide was placed onto the drop at a 45° angle and allowed to spread along its back edge, then moved forward spreading the semen drop over the surface of the first slide to make an even smear. The slide was left on the lab bench overnight to air dry at room temperature. Following the air drying period, the slide was fixed and stained according to the manufacture's guidelines (Van der Horst and Maree, 2009). The slide was immersed into a Coplin jar containing SpermBlue® fixative (SpermBlue® fixative, Microptic, S.L., Barcelona, Spain), and left undisturbed for 10 minutes. The slide was removed carefully from the Coplin jar and left upright at an angle of about 70°, allowing contact with a paper towel to drain the excess fixative. The slide was then dipped into a Coplin jar containing SpermBlue® staining solution (SpermBlue® stain, Microptic, S.L., Barcelona, Spain) and left for a 15 minutes period, after which it was gently immersed in distilled water for few seconds to remove the excess stain. The slide was then left to drain and air-dried at room temperature as described above. Following completion of the staining procedures, the slide was permanently mounted by applying a drop of non-aqueous DPX mounting medium (Dako, CA, USA) and a cover slip to the slide. Two morphology slides were prepared from each sample.

Morphological characteristics of the stained spermatozoa were evaluated by Computer Aided Sperm Morphology Analysis (CASMA) using the SCA® module. (blue filter, 100x oil immersion objective, 10x eyepiece, brightness \pm 435, contrast=100). The SCA® system equipped with a Basler A312fc digital colour camera (Microptic, S.L., Barcelona, Spain), mounted on a Nikon Eclipse 200 Microscope (IMP, Cape Town, South Africa). As shown in Figure 3.3, a total of 100 spermatozoa per semen sample from various systematically selected microscopic fields were analysed according to WHO criteria (WHO, 2010). Overlapping or clumping spermatozoa and those with heads obscured by tail or debris were excluded, as they were not possible to be analysed adequately.

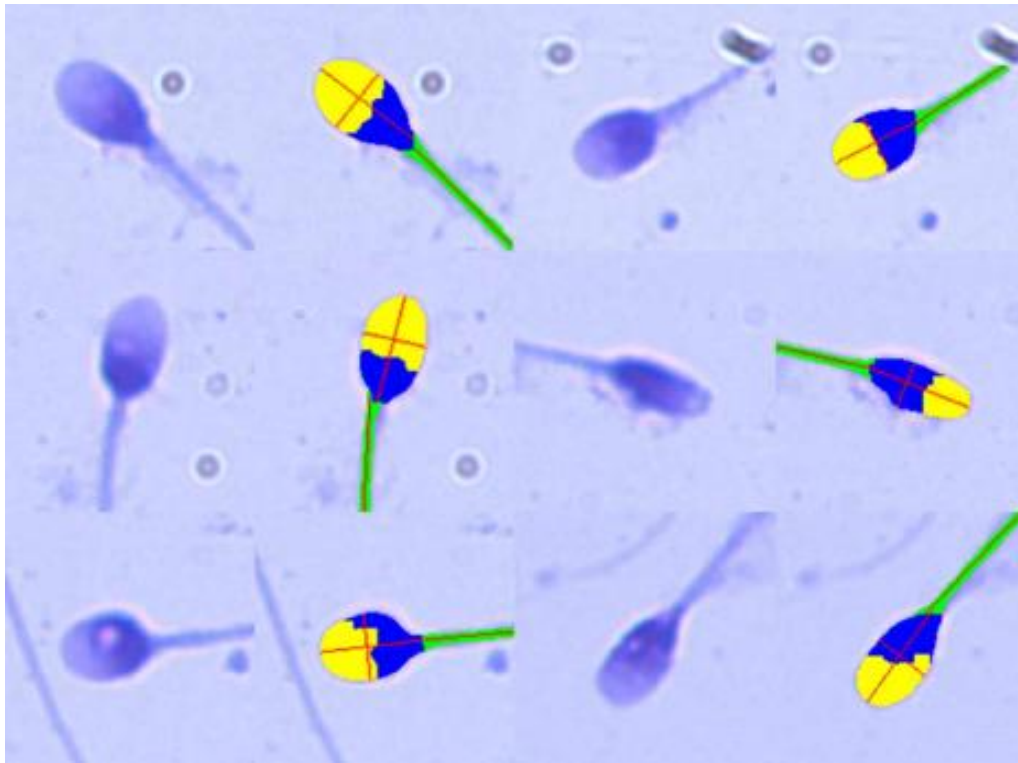


Figure 3. 3: SCA morphology analysis of spermatozoa stained with SpermBlue. Acrosome region stains yellow, postacrosomal region of the head dark blue, and the midpiece green.

3.2.8 Sperm viability

The integrity of the sperm plasma membrane was estimated by means of a dye exclusion technique (WHO, 2010), using the membrane-impermeant Eosin-Nigrosin stain (Sigma-Aldrich, St Louis, MO, USA), which penetrates only the compromised plasma membrane of dead and dying cells. A 10 μ l of diluted semen adjusted to a concentration of 5×10^6 spermatozoa/mL was added and mixed with 20 μ l Eosin and 30 μ l Nigrosin. A drop of the mixture was spread across the length of a labelled microscope slide using another slide to create a uniformly spread smear. After air drying, the slide was mounted using a DPX mounting medium (Dako, CA, USA) and covered with a cover slip. Two viability slides were made from each sample. Viability status of spermatozoa was assessed using CASA (Sperm Class Analyzer version 5.4 - SCA®, Microptic, S.L., Barcelona, Spain) equipped with a Basler A312fc digital colour camera (Microptic, S.L., Barcelona, Spain), mounted on a Nikon Eclipse 200 Microscope (IMP, Cape Town, South Africa), with bright field optics. From each sample, the viability

of 100 spermatozoa was evaluated using the counter module of the SCA[®] system (positive phase contrast observation setting; 20x objective lens; 10x eyepiece and a blue filter). The number of pink-stained (non-viable) spermatozoa and those unstained (viable) were counted in randomly selected fields. Results were reported as percentage of viable spermatozoa (Figure 3.4).

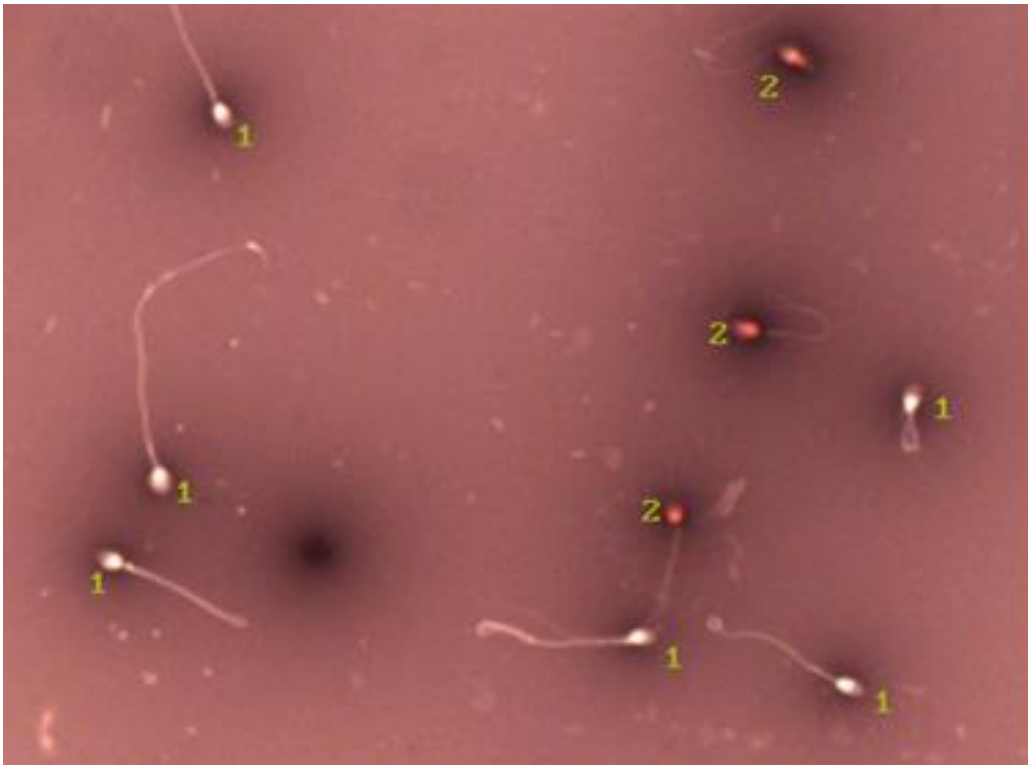


Figure 3. 4: Viability assessment of spermatozoa stained with dye exclusion, using the counter module of the SCA[®] system. Unstained cells (1) are viable; Stained cells (2) are non-viable.

3.3 Advanced semen analysis assays

3.3.1 Acrosome reaction

The acrosomal status of the spermatozoa of each sample was determined visually under fluorescent microscopy using fluorescent labelled lectins (WHO, 2010). A 5 μL drop of the semen sample adjusted to a concentration of $2 \times 10^6/\text{mL}$ was placed on a labelled frosted microscope slide and left to air dry overnight. The slide was fixed by placing it in 70 % ethanol (30 minutes, 4°C) and allowed to dry subsequently. 450 μL of Phosphate Buffered Saline (PBS) (Gibco, Scotland, UK) was added to 50 μL of Fluorescein isothiocyanate-labelled pisum sativum agglutinin (FITC-PSA) (Sigma Chemicals Co., St Louis, MO, USA) in an Eppendorf tube to provide a concentration of 125 $\mu\text{g}/\text{mL}$. 10 μL of this solution was layered on top of the fixed drop and left in a dark environment for 45 minutes. The slide was then dipped gently in distilled water to rinse the unbound FITC-PSA followed by air drying and mounting with the anti-fade fluorescent mounting medium (Dako North America, Inc.) with a cover slip was added. The slide was visualized (within less than 12 hours) with fluorescent microscopy (Nikon Corporation, Tokyo, Japan), using a green fluorescein filter at $\times 1000$ magnification and oil immersion, 510-560 nm. 200 spermatozoa were counted from various randomly selected fields with the aid of a laboratory counter. As shown in Figure 3.5, acrosome-intact [bright and uniformly green fluorescing acrosome region] and acrosome-reacted spermatozoa [no fluorescence or dull green fluorescing acrosomes] (Oyeyipo et al., 2014). Results were expressed as percentage.



Figure 3. 5: Acrosomal status of spermatozoa as seen under florescent microscope using fluorescently labelled lectins

3.3.2 DNA fragmentation

Sperm DNA fragmentation was determined by using a terminal deoxynucleotidyl transferase-mediated fluorescein-TUNEL assay with an APO-DIRECT™ kit (BD Biosciences Pharmingen, San Diego, CA, USA) as described by Sharma et al. (2013). The positive and negative control cell suspensions provided by the manufacturer were included in each run. For cell preparation and fixation, an aliquot of completely liquefied semen sample adjusted to a concentration of $2\text{--}3 \times 10^6$ spermatozoa/mL was centrifuged at $300 \times g$ for 7 minutes. The supernatant seminal plasma was gently aspirated with a Pasteur pipette and the pellet was resuspended in 1.0 mL of 3.7 % paraformaldehyde fixation buffer (10 mL of 37 % formaldehyde and 90 mL of PBS, pH 7.4) and left on ice for 30–60 minutes. The cells were then centrifuged ($300 \times g$, 5 minutes) and the pellet was resuspended in 1.0 mL of ice-cold 70 % ethanol (1×10^6 spermatozoa/mL) and stored at -20°C until the time for the TUNEL assay.

Each tube was gently vortexed for re-suspending the sperm cells settled after the storage period in ethanol. After the tubes were centrifuged ($300 \times g$, 5 minutes) to remove the ethanol, the pellet was double-washed in 1.0 mL of Washing Buffer and centrifuged ($300 \times g$, 5 minutes) to discard the supernatant. Resuspended in 1.0 mL of 0.2 % Triton X-100 5 minutes to permeabilize the cells. For

each sample, the sperm pellet was resuspended in 50 mL of the Staining Solution (10 mL Reaction Buffer, 0.75 mL Terminal Deoxynucleotidyl Transferase (TdT) Enzyme, 32.25 mL distilled water, and 8 mL FITC–Deoxyuridine triphosphate (dUTP)) and mixed thoroughly with a vortex to permeate the staining solution homogeneously into the cells. TdT enzyme, a template-dependent DNA polymerase, catalyses the incorporation of the labelled dUTP into the exposed terminal 3'–OH ends of each DNA strand, providing a direct marker of DNA breaks (Figure 3.6).

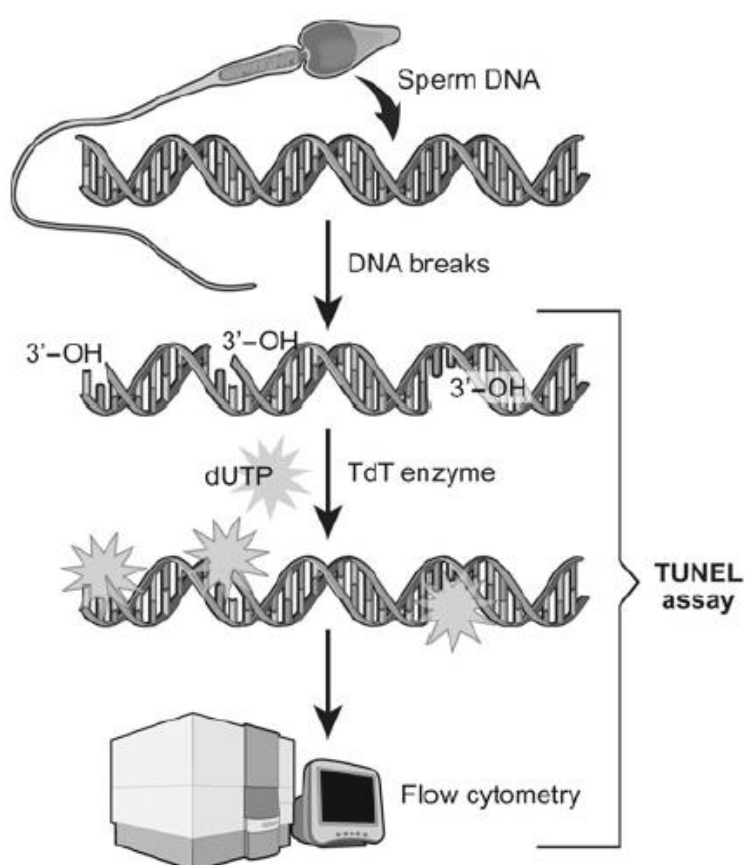


Figure 3. 6: Schematic of Sperm DNA Fragmentation Analysis Using the TUNEL Assay, (Sharma et al. 2013).

Following an incubation period of 60 minutes (37°C, 5 % CO₂), the cells were rinsed twice with 1.0 mL rinsing buffer. In each rinsing step, the mixture was centrifuged (300×g, 5 minutes) and the supernatant of each tube was removed by gentle aspiration. The sperm pellet was then resuspended in Propidium Iodide (PI)/RNase Staining Buffer (5 mg/mL PI, 200 mg/mL RNase). PI is a fluorescent counterstain, which binds to and labels all DNA, so that every spermatozoon can be counted, and the consequent proportion of the spermatozoa with DNA fragmentation can be determined. The tubes were left to incubate at room temperature for 30 minutes in a darkened environment. Upon completion of the staining procedure, spermatozoa within the PI/RNase solution were analysed by flow cytometry.

3.3.3 Intracellular Superoxide (O₂⁻)

For the evaluation of ROS levels in spermatozoa, intracellular O₂⁻ was quantified using dihydroethidium (DHE) as an oxidative fluorescent probe. An allocated volume of each liquefied ejaculate was placed in a pre-warmed (37°C) 15 mL plastic Falcon tube. The samples underwent a wash in an equal volume of pre-prepared HAMS-BSA (Human Albumin Serum [Sigma Chemicals Co., St Louis, MO, USA]–Bovine Serum Albumin [Roche Diagnostics GmbH, Mannheim, Germany]). Following the centrifugation period (300×g, 10 minutes), the sperm pellet was washed twice in 3 mL of HAMS-BSA (300×g, 5 minutes). After the second wash, the supernatant was aspirated carefully and discarded. The isolated pellet was resuspended in 2 mL of pre-warmed (37°C) HAMS-F10 medium and its final concentration was adjusted to 5 × 10⁶ cells/mL. The obtained sperm suspension was divided equally into two aliquots labelled as unstained and DHE stained. 1µL of distilled water was added to the unstained tube, while the stained tube was exposed to 1µL of DHE. The samples were incubated for 15 minutes (37 °C, 5 % CO₂, 95 % humidity) and subsequently resuspended in 2 mL of PBS. The tubes were mixed gently and then centrifuged at 300×g, 20°C for 5 minutes. 100 µL were pipetted carefully from the pellet of each tube and were transferred to a disposable 5 mL polystyrene round-bottom tube (BD Falcon™ 352052, BD Biosciences, USA). The

cells in each tube were then re-suspended in 900 mL of pre-warmed (37°C) PBS and subsequently analysed using flow cytometry.

3.3.3.1 Flow cytometry analysis

The fluorescence signals of labelled spermatozoa were assessed by means of flow cytometry (Becton Dickinson FACSCalibur analyser, Life Technologies), using an argon laser with excitation at 488nm and emission wavelength 530 nm. For each assay, a total of 20,000 gated events were examined at a flow rate of < 100 cells/second. The sperm population was appropriately gated using forward and side (90 degrees) light scatter signals so as to eliminate debris and non-sperm particles, and thus excluding their effects on overall fluorescence (Figure 3.7 A and B). The output data was then imported and analysed using Flowjo© V10 software (FlowJo, Ashland, Ore. USA). Fluorescence signals were represented on a histogram after logarithmic amplification.

For the assessment of DNA fragmentation, green (FITC-dUTP) fluorescence intensity was measured in the FL-1 channel (530/30 nm bandpass filter, which can detect a range of wavelength between 515–545 nm), and red (PI) fluorescence was set to be measured in the PE channel (585/42nm bandpass filter, which detects a range of wavelength between 564–606 nm). The intensity of PI and FITC fluorescence was quantified as median fluorescence intensity represented by a frequency histogram (Figure 3.7 C and D). Data were expressed as percentage of DNA fragmented spermatozoa. For the quantification of sperm intracellular $O_2^{\cdot-}$, red fluorescence emissions of DHE were detected in FL-2 channel. Logarithmic amplification, fluorescence signals were represented on a histogram (Figure 3.8). Data were expressed as median DHE fluorescence intensity (MFI).

A three-colour Calibrite kit (BD Bioscience–340486) with separate APC beads (BD Bioscience – 340487) was used for daily monitoring of instrument performance by adjusting the instrument settings to set fluorescence compensation and to check instrument sensitivity.

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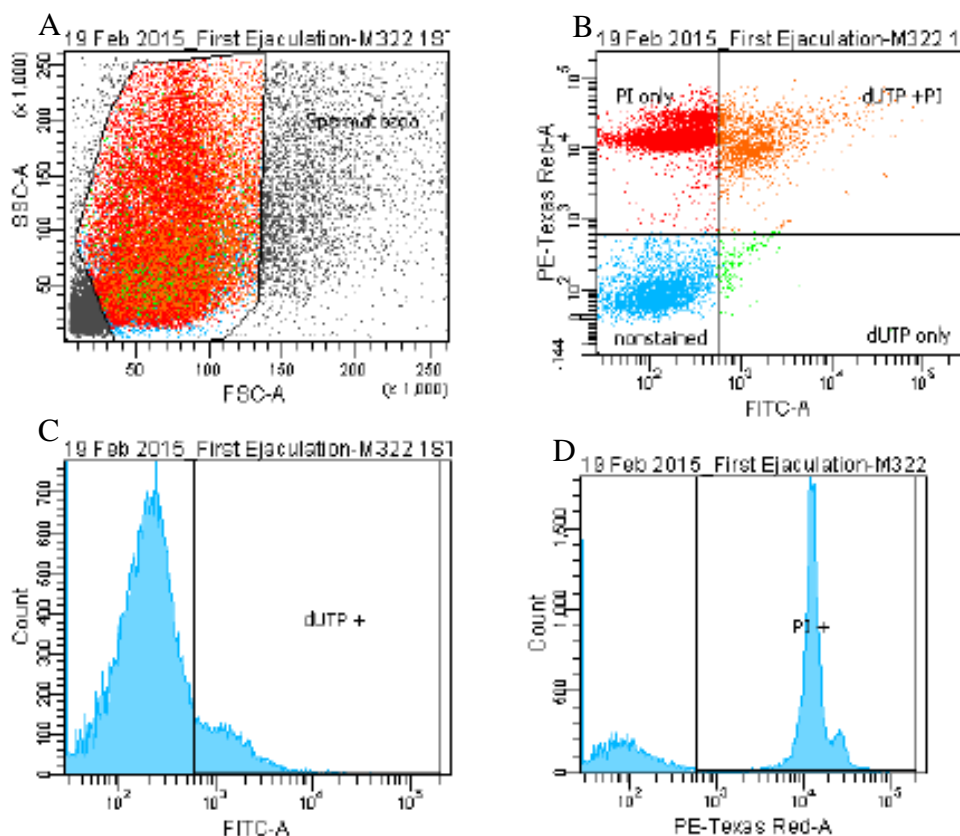


Figure 3. 7: Flow cytometry plots (dot plots and histograms). A. depicts a forward vs. scatter plot [FSC vs. SSC] containing all interrogated events. The sperm population was gated based on the resulting aggregation of events of similar cell size (FSC) and granularity (SSC). B. Shows a dot plot of the gated sperm population separated according to Propidium Iodide (PI) and Fluorescein isothiocyanate (FITC) binding. A quadrant was inserted to separate sperm sub-populations, where PI stains necrotic and early necrotic cells while Terminal deoxynucleotidyl transferase (dUTP) (coupled to FITC probe) binds to cells with fragmented DNA. The lower left quadrant represents unstained viable cells. C and D. represent histogram plots, showing positive and negative populations for dUTP-FITC and PI probes respectively.

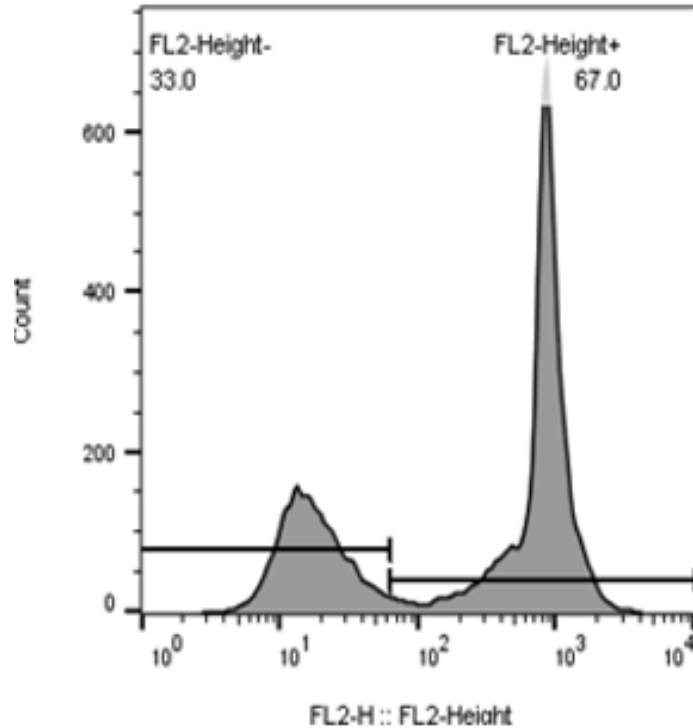


Figure 3. 8: Flow cytometry histogram generated by measuring the sperm intracellular O_2^- levels. The count represents the different numbers of spermatozoa. O_2^- negative spermatozoa are without fluoresce detected in channel FL2-Height-, and are located on the left side. O_2^- positive cells emit fluoresce in channel FL2-Height+, and located on the right side.

3.3.4 Seminal plasma TBARS and antioxidant assays

3.3.4.1 Sample preparation

In preparation for the assessment of seminal plasma TBARS levels and antioxidant activity, semen samples were centrifuged (300xg, 10 minutes at room temperature) and the seminal plasma of each sample was pipetted carefully and transferred into a labelled cryopreservation tube (2 mL) and then stored in liquid N⁺ (-196°C) until time of analysis.

3.3.4.2 TBARS assay

The TBARS assay provides an overall measure of malonicdialdehyde, which is a reactive compound produced during lipid peroxidation caused by ROS (Moselhy et al., 2013). TBARS was assessed by means of spectrophotometric analysis using a SPECTRA-max PLUS-384 spectrophotometer with SoftMax® Pro 4.8 software (Molecular Devices Corporation, Labotec Industrial Technologies, Cape Town, South Africa) for data acquisition and analysis. Molar extinction coefficient (1.54 x 10⁵M/cm) was used to calculate concentrations.

TBARS levels in seminal plasma were measured according to the method described by Jentsch et al. (1996). In preparation for the assay, seminal plasma samples were removed from the liquid N⁺ and allowed to thaw at room temperature. 200 µl of the seminal plasma were aspirated and added to a mixture of 25 µl butylated hydroxytoluene (4 mM) (Fluka Chemie, Buchs, Switzerland) in ethanol (Merck Chemicals, Cape Town, South Africa) and 200 µl (0.2 M) orthophosphoric acid (Sigma-Aldrich, Cape Town, South Africa) in 2 mL Eppendorf tube and mixed thoroughly on a vortex mixer for 10 seconds. 25 µl of Thiobarbituric acid (TBA) (Sigma-Aldrich, Cape Town, South Africa) reagent (0.11M of TAB dissolved in 0.1M sodium hydroxide) was added to the mixture, and the tube was vortexed again. Following the incubation period (90°C, 45 minutes) in a heating block, the reaction was terminated by immersing the tubes in ice for 2 minutes, and left afterward at room temperature for 5 minutes. TBARS was extracted by the addition of 500 µl n-butanol (Merck Chemical, Cape Town, South Africa), and 50 µL saturated sodium chloride to each sample. After

that, the tubes were vortexed and then centrifuged (13000xg, 1 minute). 250 μ l from the supernatant upper butanol phase was carefully pipetted into an empty well, and the absorbance was read at 532 nm wavelength. Values were expressed in μ mol/L of seminal plasma.

3.3.4.3 Catalase (CAT) activity assay

The principle of this assay is based on the ability of CAT, a ubiquitous antioxidant enzyme, to reduce H_2O_2 into H_2O and molecular oxygen (Iwase et al., 2013). CAT activity was measured according to the method described by Aebi (1984). For this assay, seminal plasma samples were removed from the liquid N^+ and thawed at room temperature. Seminal plasma was then diluted 1:10 in 50 mM phosphate buffer (pH 7.0), after which 0.01 mL was added to each well of a Costar (Corning) 96-well, UV-transparent plate (Sigma-Aldrich, Cape Town, South Africa). 220 μ L of freshly prepared 40 mM H_2O_2 (Sigma Aldrich, Cape Town, South Africa) was subsequently added to each well. The rate of decomposition of H_2O_2 was measured by spectrophotometer (SPECTRAMaxPLUS-384, Molecular Devices, San Francisco, CA, USA) based on changes in absorbance at 240 nm. CAT activity was expressed as units/mg protein.

3.3.4.4 Superoxide dismutase (SOD) activity assay

SOD, a vital enzymatic antioxidant, plays a critical protective role by catalyzing the dismutation of $\text{O}_2^{\cdot-}$ to H_2O_2 . SOD activity was measured using the SOD Assay Kit-WST (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. This assay is based on the principle that water-soluble tetrazolium salt (WST-1) reduces $\text{O}_2^{\cdot-}$ causing a colour changes due to the formation of a water-soluble formazan dye. SOD activity was therefore measured by quantifying the decrease in colour development as a result of the reduction in $\text{O}_2^{\cdot-}$ by WST-1. Sample analysis was performed in triplicate.

After thawing, 20 μ l of sample solution was added to each sample well, as well as the second blank well (blank 2). 20 μ l of dH_2O was added to the first (blank 1) and the third blank well (blank 3). 200 μ l of the WST working solution was added to each well, and agitated to allow for adequate mixing.

20 µl of dilution buffer was added to the second and the third blank well, with 20 µl of Enzyme Working Solution added to each sample well and the first blank well. The contents of the well were mixed adequately before being incubated at 37 °C for 20 minutes. The absorbance was immediately read at 450 nm using the microplate reader (SPECTRAmaxPLUS-384, Molecular Devices, San Francisco, CA, USA). SOD activity was then calculated using the equation below. Values were expressed as units per mg protein (U/mg protein).

$$\text{SOD activity (inhibition rate \%)} = \frac{[(\text{blank 1} - \text{blank 3}) - (\text{sample} - \text{blank 2})]}{(\text{blank 1} - \text{blank 3})} \times 100$$

3.4 Statistical analysis

3.4.1 Aim I: Effect of abstinence period on semen quality.

- Data were checked for normal distribution and statistical comparisons between short and long abstinence periods were performed using Paired Student's t-tests on GraphPad Prism™ software (GraphPad™ Software, Version 6.0, San Diego, CA, USA). Box and Whiskers Plots were used to graphically display differences in semen values between short and long abstinence periods. Data were presented as Mean \pm SD. Statistical significance was set at $p < 0.05$.

Qualitative multivariate analyses were carried out by means of Statgraphics software (version XVII, Centurion), after which grouped quantitative parameters were visually depicted using Star Glyphs (Fienberg, 1979) to represent variances and similarities between short and long abstinent periods.

Star Glyphs and Sunray Plots have been utilised to visualize consistent variances between the short and long abstinence periods by combining groups of relevant input parameters per symbol. Each arm of the star plot symbolizes a particular semen variable, with comparative rather than absolute differences represented. The variable of interest with the lowest mean between short and long abstinence periods is represented as 10 % of the length of the arm, whereas the variable with the higher mean is represented as 100 %. The length of the coloured area along each axis reveals the pattern of change in each value, relative to the maximum value, for the variable of interest. The selected multivariate groupings of semen parameters displayed considerable differences in the patterns between the two abstinence periods and thereby were considered suitable for star symbol analysis. Basic semen, motility, kinematic and advanced parameter groupings are displayed in Figures 4.2, 5, 7 and 9.

- Prediction of various basic and advanced semen parameters of the second ejaculate from a set of basic semen parameters of the first ejaculate was performed using a multiple linear

regression model. Best subsets regression was used to search for subsets of predictors (independent variable) that best predict the dependent variable with a reasonable proportion of variance (R^2). DellTM StatisticaTM data analysis software system, version 13 (StatSoft Inc.), as a statistical package, was used to conduct the analysis. The independent (predictor) variables include a set of basic semen parameters obtained from the first ejaculate, i.e. semen volume (mL), pH, T.S.C. (10^6 /ejaculate), concentration (10^6 /mL), total motility (%), progressive motility (%), normal morphology (%), viability (%), VCL ($\mu\text{m/s}$), VSL ($\mu\text{m/s}$), VAP ($\mu\text{m/s}$), LIN (%), WOB (%), ALH (μm), BCF (Hz). The dependent variables include: sperm concentration (10^6 /mL), total motility (%), progressive motility (%), normal morphology (%), viability (%), DNA fragmentation (%), sperm O_2^- (MFI), TBARS ($\mu\text{mol/L}$), CAT (U/mL) and SOD (U/mg protein) of the second ejaculate.

Once the subset was determined, a multiple linear regression model was fitted and a standard regression coefficient was also calculated to facilitate comparison between the variables within the subset. R^2 and cross validated (CV) R^2 were reported. Statistical significance was set at $p < 0.05$. The model equation employed has the following form:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_k X_k$$

Y = Dependent variable, X = Independent variable, β_0 = Estimated intercept, $\beta_1, \beta_2, \beta_k$ = Estimated slope coefficients.

3.4.2 Aim II: Relationship between advanced and basic semen parameters

- For the determination of the correlations between the basic semen characteristics and advanced functional parameters, a Spearman rank correlation coefficient (r) was used. To conduct the analysis, Statistica 13 was used as a statistical package. Statistical significance was set at $p < 0.05$. The sample size (n) was determined to be the number of both first and second ejaculates and analyzed per advanced parameter.

Multiple Factor Analysis was used to visually depict correlations between each advanced variable (arrows with blue lettering) and a set of basic semen variables (arrows with red lettering). Basic semen variables, which have arrows pointing roughly in the same direction relevant to the advanced variables have positive correlations, while arrows pointing in the opposite directions are negatively correlated. Similarly, arrows perpendicular to the direction of the advanced variable arrow indicates no correlation. Ideally, the arrows should stretch into the outer circle to be considered significant.

- Receiver Operating Characteristic (ROC) curves were employed for establishing cut-off values for advanced semen parameters from a number of basic semen parameters [e.g. Motility (%), progressive motility (%), viability (%)] with WHO defined reference values. ROC calculations were done using the “pROC” package in R while the sensitivity, specificity and the area under the curve (AUC) of the test were reported. Statistical significance was set at $p < 0.05$.

Chapter 4: Results

The data collected during this study is reported in this chapter. Results were statistically analysed according to the aims and objectives of the study and consequently presented by means of relevant tables and figures for better comparisons.

4.1 Aim I: Effect of abstinence period on semen and sperm quality.

The statistically analyzed data obtained from Aim I of the study, in which basic and advanced semen parameters were compared between the short and long abstinence periods, will be presented in the following section.

4.1.1 Influence of abstinence period on basic semen parameters

As shown below (Table 4.1), semen volume was significantly lower in samples collected after short abstinence period compared to those collected after long abstinence (2.01 ± 0.09 vs. 3.30 ± 0.14 ; $p < 0.0001$). Semen pH was significantly higher after short abstinence (7.69 ± 0.02 vs. 7.58 ± 0.02 ; $p = 0.0001$), while no significant difference was observed in semen viscosity (4.34 ± 0.08 vs. 4.44 ± 0.08 ; $p = 0.0650$). Both T.S.C. (85.75 ± 63.43 vs. 197.0 ± 158.8 ; $p = 0.0001$) and sperm concentration (43.95 ± 26.03 vs. 60.34 ± 41.42 ; $p = 0.0001$) were significantly decreased after short abstinence compared with long abstinence periods. No significant differences were observed with regards to the percentages of viability (67.34 ± 7.643 vs. 68.98 ± 7.833 ; $p = 0.0705$) and normal morphology (17.45 ± 6.288 vs. 17.41 ± 6.551 vs.; $p = 0.4707$) (Figure 4.1 A–G). Star symbol Plots representing the trend of differences for basic semen characteristics between short and long abstinence periods are visualized in Figure 4.2.

Table 4. 1: Semen characteristics in short vs. long abstinence (n=100). Data are presented as Mean±SD.

Parameter	Long abstinence	Short abstinence	P value
Volume (mL)	3.30± 0.14	2.01±0.09	< 0.0001
pH	7.58±0.02	7.69±0.02	< 0.0001
Viscosity (cP)	4.44±0.08	4.34±0.08	0.0650
T.S.C. (10 ⁶ /ejac)	197.0±158.8	85.75±63.43	< 0.0001
Concentration (10 ⁶ /mL)	60.34±41.42	43.95±26.03	< 0.0001
Viability (%)	68.98±7.833	67.34±7.643	0.0705
Morphology (%)	17.41±6.551	17.45±6.288	0.4707

cP = Centipoise, T.S.C. = Total Sperm Count.

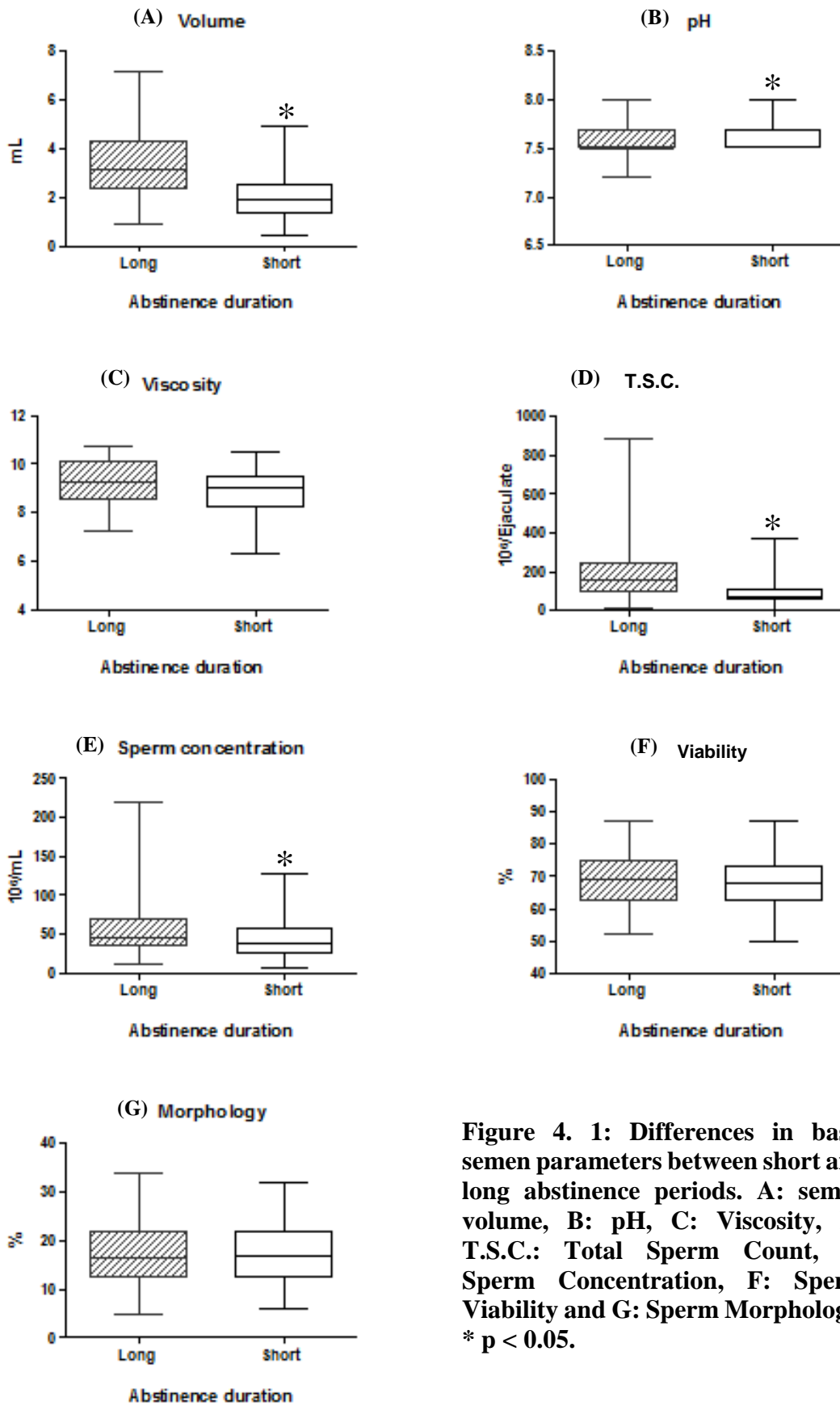


Figure 4. 1: Differences in basic semen parameters between short and long abstinence periods. A: semen volume, B: pH, C: Viscosity, D: T.S.C.: Total Sperm Count, E: Sperm Concentration, F: Sperm Viability and G: Sperm Morphology. * p < 0.05.

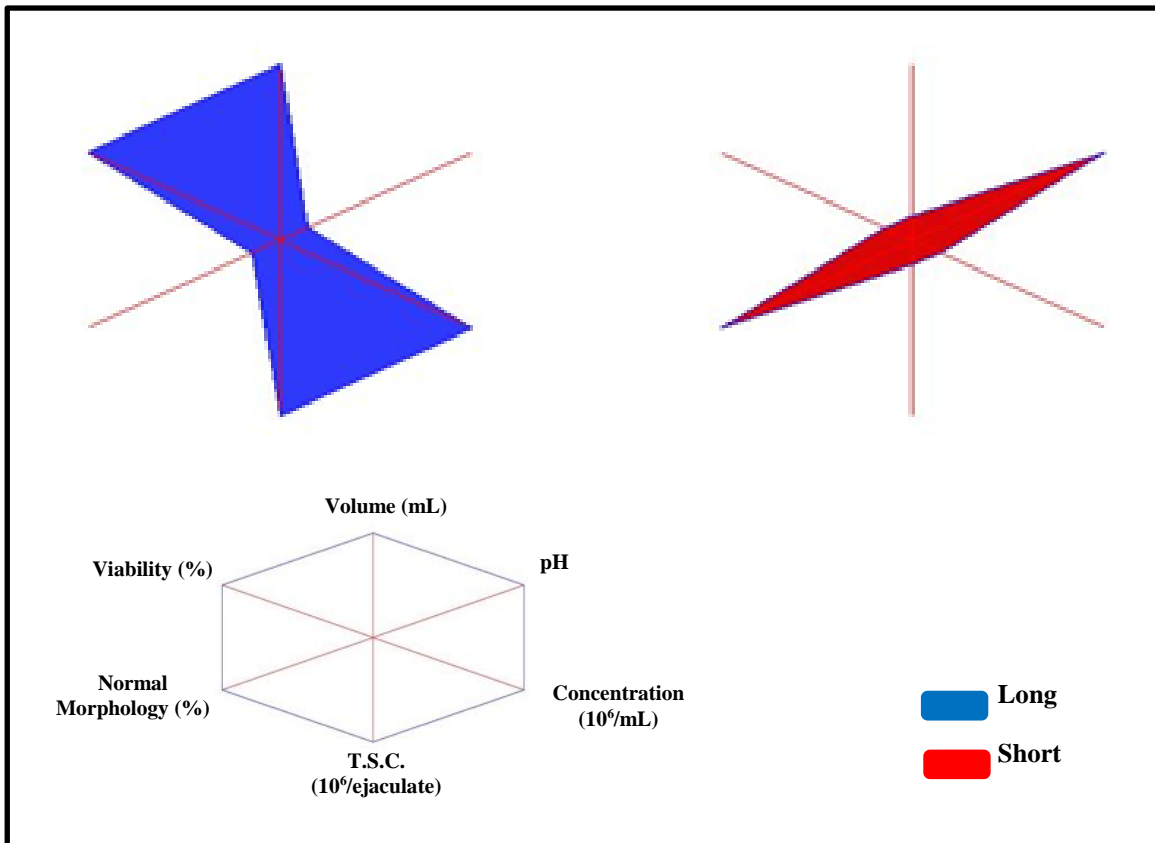


Figure 4. 2: Star symbol plots comparing six basic semen parameters between short and long periods of abstinence.

Results in Table 4.2 illustrate a significant increase in the percentages of total motility (62.29 ± 15.33 vs. 58.86 ± 15.10 ; $p = 0.0013$), progressive motility (49.58 ± 14.74 vs. 44.98 ± 13.67 ; $p = 0.0001$) and type a spermatozoa (14.03 ± 7.055 vs. 11.17 ± 6.560 ; $p = 0.0001$) in the samples collected after short abstinence compared with those collected after long abstinence periods. The percentages of type c (12.72 ± 3.898 vs. 13.85 ± 3.580 ; $p = 0.0020$) and type d (37.71 ± 15.34 vs. 41.16 ± 15.06 ; $p = 0.0012$) spermatozoa were significantly lower after short abstinence period, whereas no significant difference between short and long abstinence periods was observed in the percentage of type b spermatozoa (35.55 ± 14.56 vs. 33.81 ± 12.23 ; $p = 0.0560$) (Figure 4.3).

Table 4. 2: Sperm motility characteristics in short vs. long abstinence (n=100). Data are presented as Mean \pm SD.

Semen parameter	Short abstinence	Long abstinence	P value
Total motility (%)	62.29 ± 15.33	58.86 ± 15.10	0.0013
Progressive motility (%)	49.58 ± 14.74	44.98 ± 13.67	< 0.0001
Fast progressive [type a] (%)	14.03 ± 7.055	11.17 ± 6.560	< 0.0001
Slow progressive [type b] (%)	35.55 ± 14.56	33.81 ± 12.23	0.0560
Non-progressive [type c] (%)	12.72 ± 3.898	13.85 ± 3.580	0.0020
Immotile [type d] (%)	37.71 ± 15.34	41.16 ± 15.06	0.0012

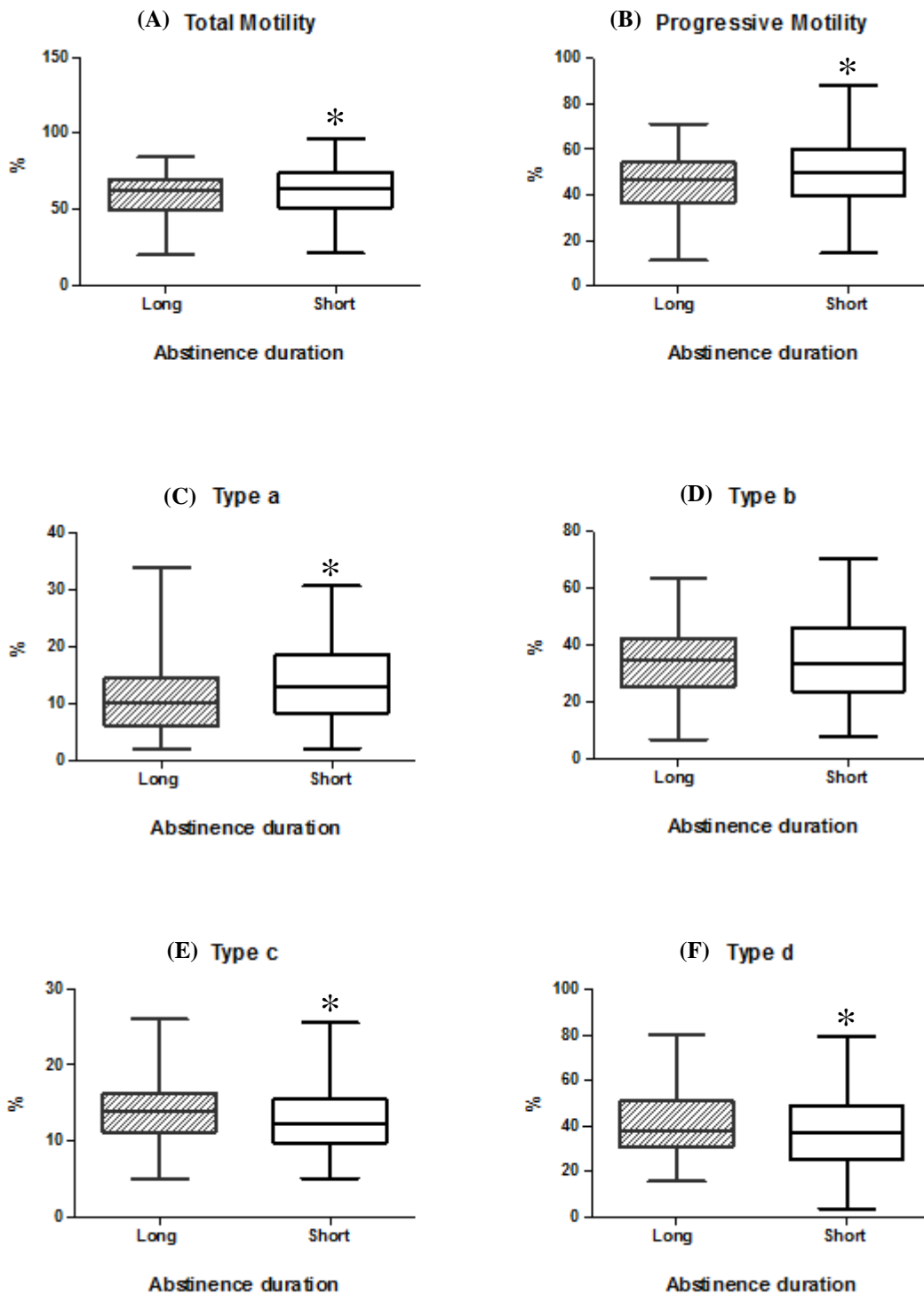


Figure 4. 3: Differences in sperm motility parameters between short and long abstinence periods. A: Sperm Total Motility, B: Progressive Motility, C: Type a, D: Type b, E: Type c, F: Type d. * p < 0.05.

Table 4.3 shows that the percentage of spermatozoa with rapid velocity was significantly higher after short abstinence compared with long abstinence (48.58 ± 14.97 vs. 43.95 ± 13.72 $p = 0.0001$). No significant difference was observed in the percentage of spermatozoa with medium velocity between short and long abstinence periods (6.067 ± 2.395 vs. 6.412 ± 2.210 ; $p = 0.0851$). The percentage of slowly motile spermatozoa (7.647 ± 2.644 vs. 8.462 ± 2.636 ; $p = 0.0016$) was significantly lower after short abstinence (Figure 4.4). The trend of changes in sperm motility characteristics between short and long abstinence periods are illustrated in Figure 4.5.

Table 4. 3: Sperm velocity parameters in short vs. long abstinence (n=100). Data are presented as Mean \pm SD.

Parameter	Short abstinence	Long abstinence	P value
Rapid (%)	48.58 ± 14.97	43.95 ± 13.72	< 0.0001
Medium (%)	6.067 ± 2.395	6.412 ± 2.210	0.0851
Slow (%)	7.647 ± 2.644	8.462 ± 2.636	0.0016

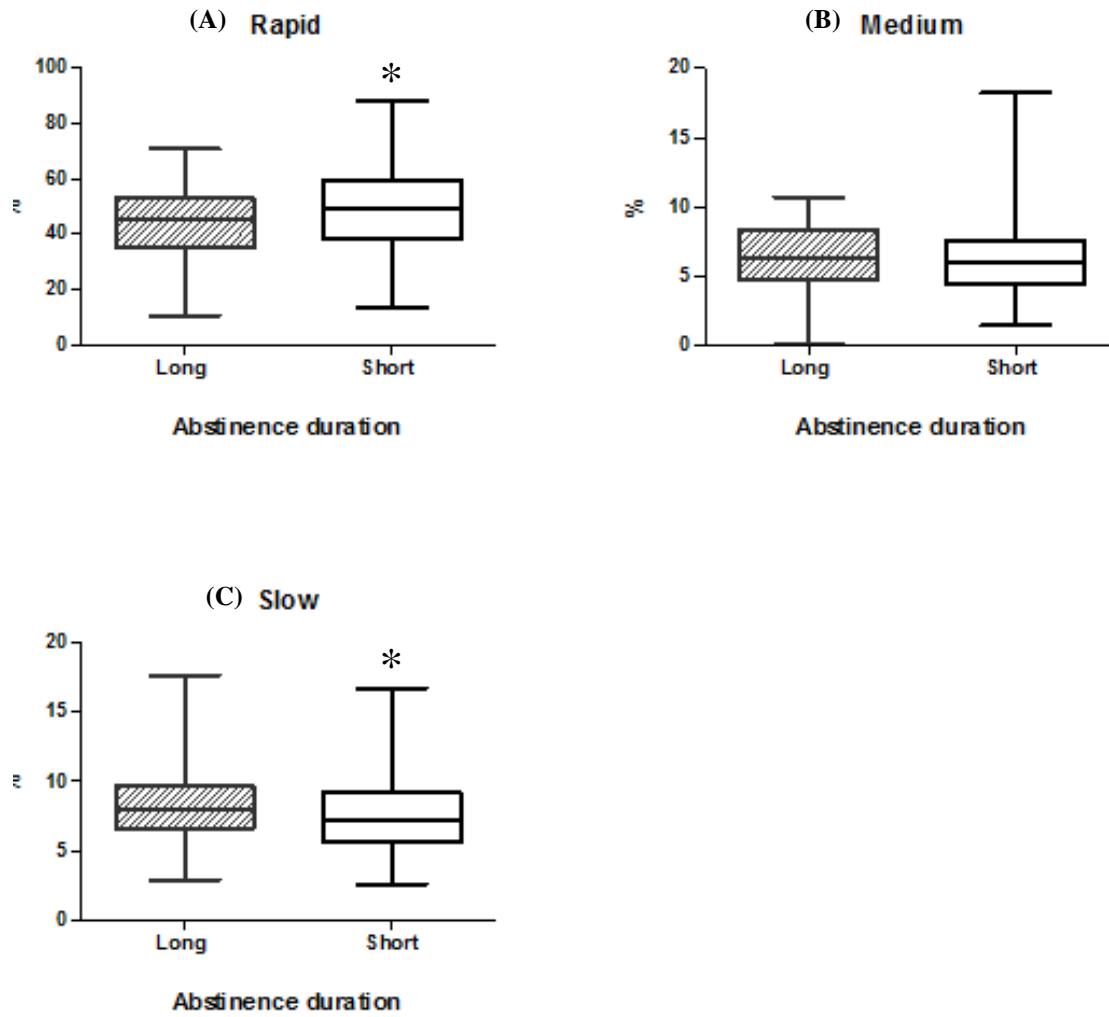


Figure 4. 4: Differences in sperm velocity parameters between short and long abstinence periods. A: Rapid, B: Medium, C: Slow. * $p < 0.05$.

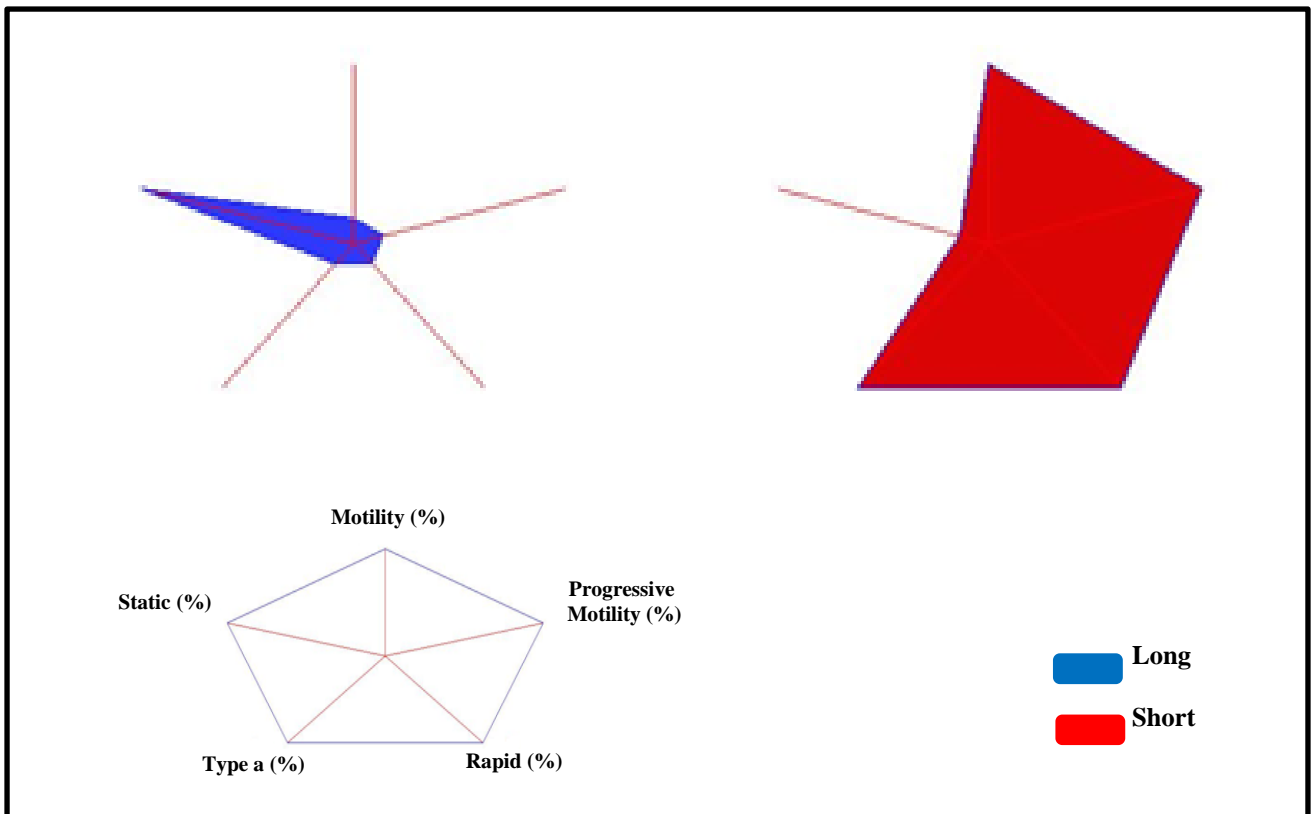


Figure 4. 5: Star symbol plots comparing five sperm motility parameters between short and long periods of abstinence.

Results pertaining to the effect of abstinence period on sperm kinematics are displayed in Table 4.4. Significant increases in VCL (81.99 ± 16.83 vs. 76.24 ± 10.82 ; $p = 0.0001$), VSL (32.90 ± 6.140 vs. 29.81 ± 5.041 ; $p = 0.0001$), VAP (53.40 ± 8.252 vs. 49.50 ± 5.891 ; $p = 0.0001$), LIN (40.89 ± 7.953 vs. 39.42 ± 5.955 ; $p = 0.0055$), STR (61.80 ± 8.185 vs. 60.24 ± 6.956 ; $p = 0.0132$) and BCF (14.87 ± 1.866 vs. 13.55 ± 2.443 ; $p = 0.0001$) were observed after short abstinence compared with long abstinence periods. No significant differences were observed between the two abstinence periods with regards to ALH (1.975 ± 0.3901 vs. 1.951 ± 0.3549 ; $p = 0.2202$) and WOB (65.83 ± 5.546 vs. 65.24 ± 4.205 ; $p = 0.0601$) (Figure 4.6 A–H). The trend of changes in sperm kinematic characteristics after short and long abstinence periods are shown in Figure 4.7.

Table 4. 4: Sperm kinematic parameters in short vs. long abstinence (n=100). Data are presented as Mean±SD.

Parameter	Short abstinence	Long abstinence	P value
VCL ($\mu\text{m/s}$)	81.99 ± 16.83	76.24 ± 10.82	< 0.0001
VSL ($\mu\text{m/s}$)	32.90 ± 6.140	29.81 ± 5.041	< 0.0001
VAP ($\mu\text{m/s}$)	53.40 ± 8.252	49.50 ± 5.891	< 0.0001
LIN (%)	40.89 ± 7.953	39.42 ± 5.955	0.0055
STR (%)	61.80 ± 8.185	60.24 ± 6.956	0.0132
WOB (%)	65.83 ± 5.546	65.24 ± 4.205	0.0601
ALH (μm)	1.975 ± 0.3901	1.951 ± 0.3549	0.2202
BCF (Hz)	14.87 ± 1.866	13.55 ± 2.443	< 0.0001

VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency, μm = micrometre, s = second, Hz = hertz.

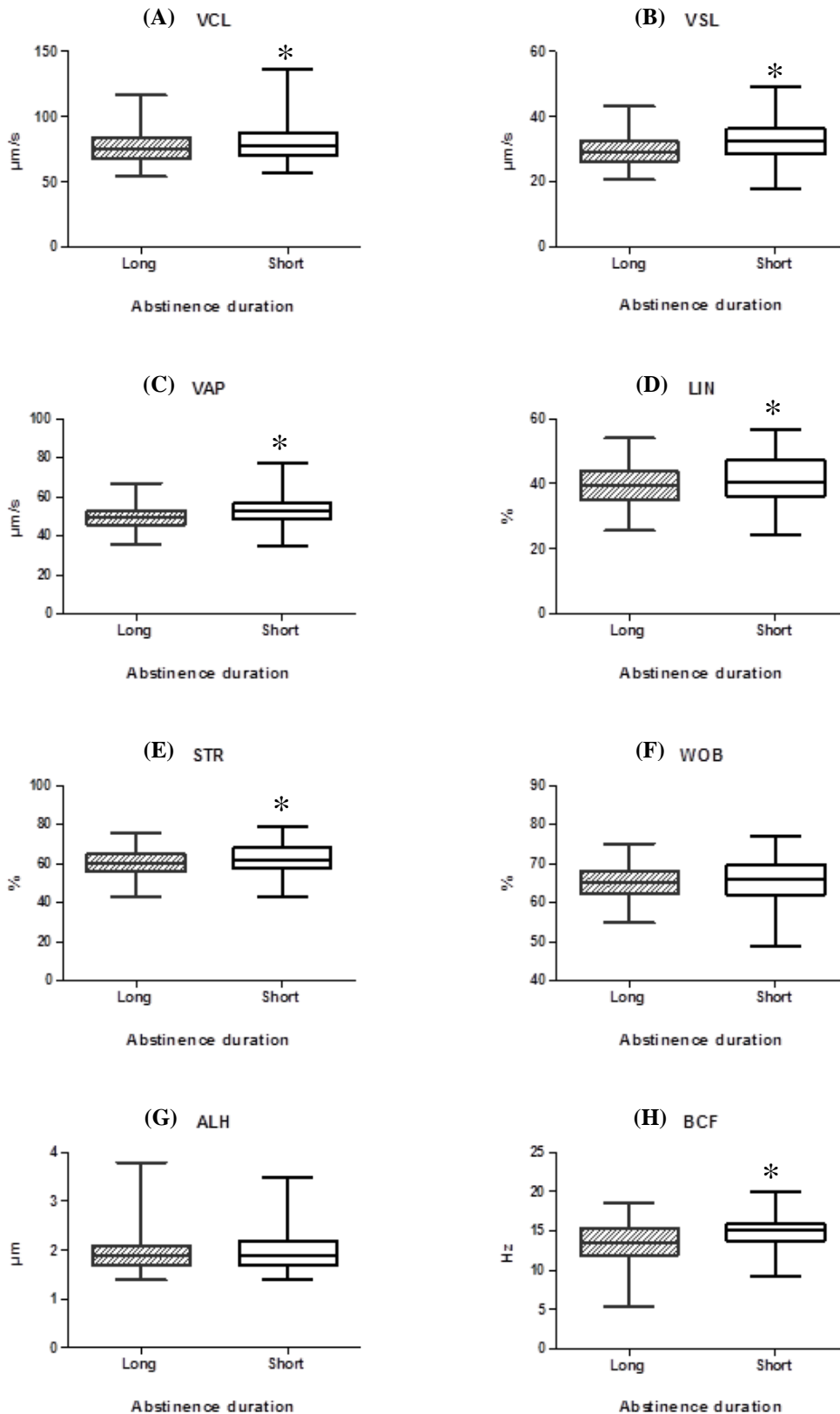


Figure 4. 6: Differences in sperm kinematic parameters between short and long abstinence periods. A: VCL (Curvilinear velocity), B: VSL (Straight-line velocity), C: VAP (Average path velocity), D: LIN (Linearity), E: STR (Straightness), F: WOB (Wobble), G: ALH (Amplitude of lateral head displacement), H: BCF (Beat-cross frequency). μm = micrometre, s = second, Hz = hertz. * $p < 0.05$.

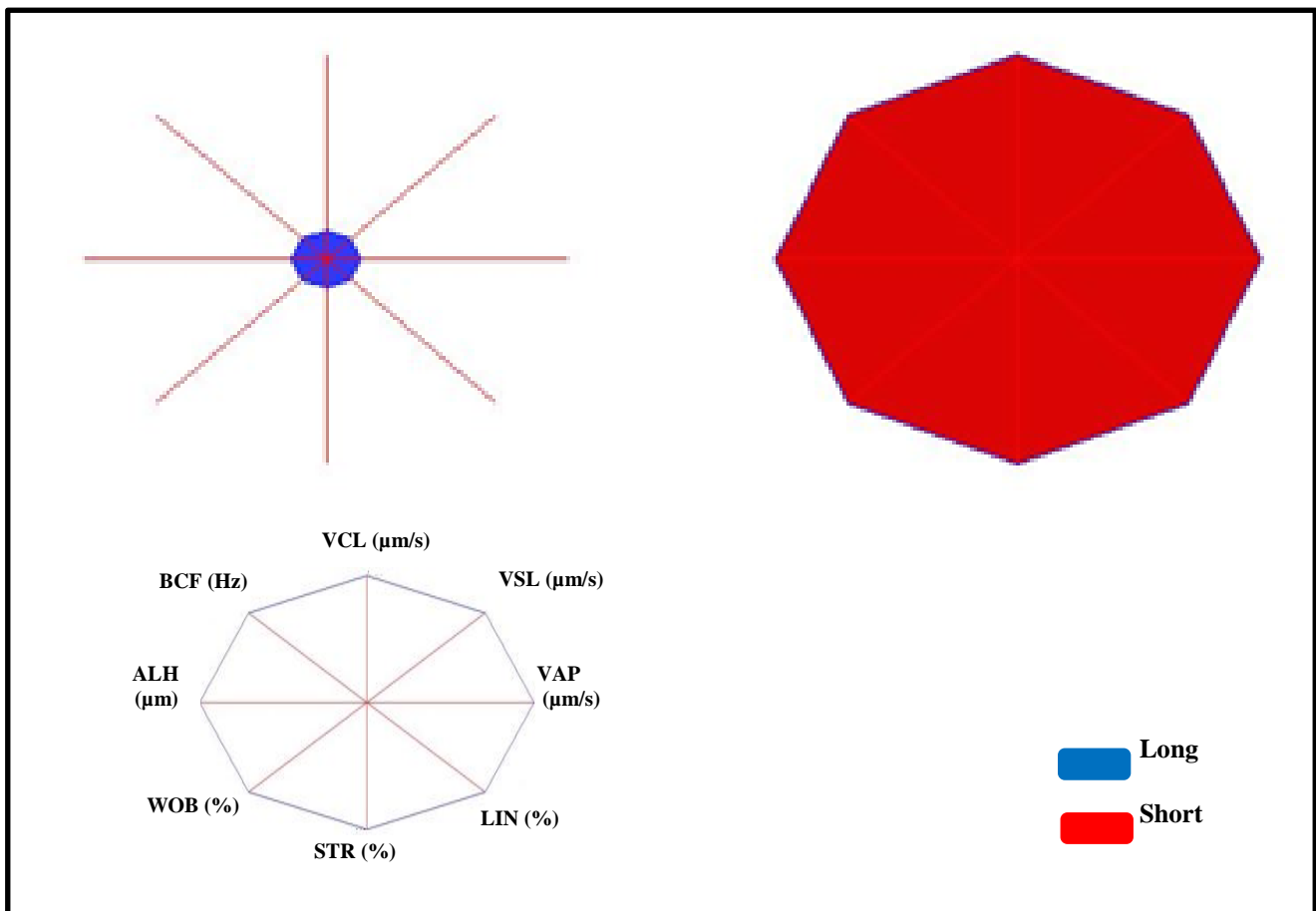


Figure 4. 7: Star symbol Plots comparing eight kinematic parameters between short and long periods of abstinence. VCL = Curvilinear velocity, VSL = Straight-line velocity, VAP = Average path velocity, LIN = Linearity, STR = Straightness, WOB = Wobble, ALH = Amplitude of lateral head displacement, BCF = Beat-cross frequency, μm = micrometre, s = second, Hz = hertz.

4.1.2 Influence of abstinence period on advanced semen parameters

Results in Table 4.5 show that the percentage of acrosome-intact spermatozoa (84.95 ± 2.523 vs. 85.60 ± 2.854 ; $p = 0.0994$), the percentage of DNA fragmentation (12.68 ± 4.979 vs. 12.85 ± 5.226 ; $p = 0.3881$), sperm intracellular $O_2^{\cdot-}$ levels ($152.6.4 \pm 118.3$ vs. 160.3 ± 95.76 ; $p = 0.3416$) as well as seminal plasma TBARS levels (23.80 ± 18.37 vs. 29.09 ± 18.47 ; $p = 0.0607$) were not significantly different between the short and long abstinence periods. Seminal plasma CAT activity (36.97 ± 10.68 vs. 34.25 ± 9.853 ; $p = 0.1030$) was also not significantly different between the two abstinence periods, while the SOD activity (4.312 ± 1.526 vs. 3.639 ± 1.277 ; $p = 0.0227$) was significantly higher after short abstinence compared with long abstinence (Figure 4.8 A–F). Star symbol plots indicating the trend of changes in advanced semen parameters between short and long abstinence periods are showed in Figure 4.9.

Table 4. 5: Advanced semen parameters in short vs. long abstinence. Data are presented as Mean \pm SD.

Parameter	Short abstinence	Long abstinence	P value
Acrosome-intact (%) (<i>n</i> =20)	$84.95.05 \pm 2.523$	85.60 ± 2.854	0.0994
DNA fragmentation (%) (<i>n</i> =20)	12.68 ± 4.979	12.85 ± 5.226	0.3881
Sperm $O_2^{\cdot-}$ (MFI) (<i>n</i> =20)	$152.6.4 \pm 118.3$	160.3 ± 95.76	0.3416
TBARS (μ mol/L) (<i>n</i> =22)	23.80 ± 18.37	29.09 ± 18.47	0.0607
SOD (U/mg protein) (<i>n</i> =22)	4.312 ± 1.526	3.639 ± 1.277	0.0227
CAT (U/mL) (<i>n</i> =22)	36.97 ± 10.68	34.25 ± 9.853	0.1030

$O_2^{\cdot-}$ = superoxide anion, MFI = median DHE fluorescence intensity, TBARS = thiobarbituric acid reactive substances, SOD = superoxide dismutase. CAT = catalase, MFI = median florescent intensity, μ mol = micromole, L = litre, U = unite, mg = milligram, mL = millilitre.

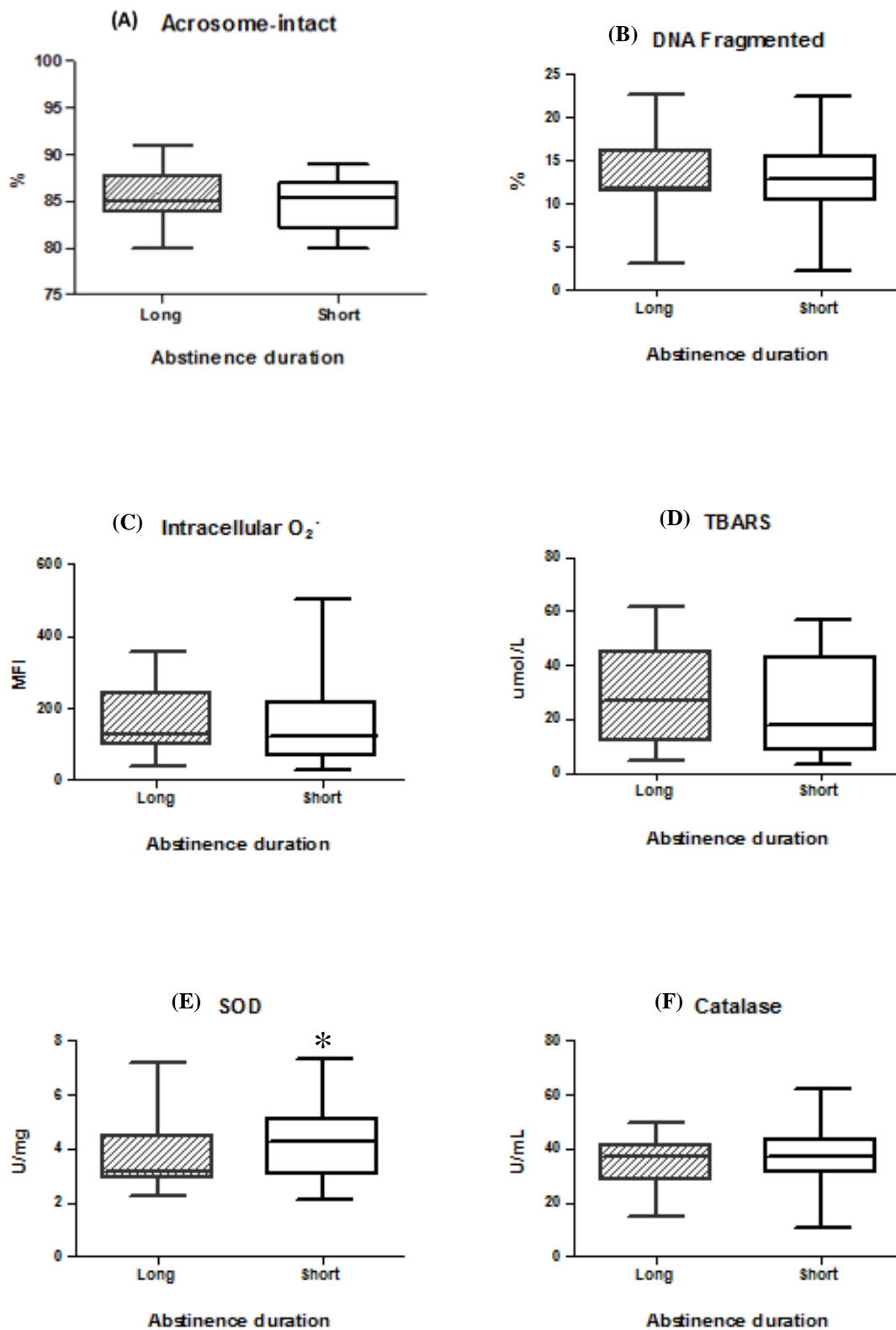


Figure 4. 8: Differences in Advanced Semen Parameters between short and long abstinence periods. A: Acrosome-intact, B: DNA fragmentation, C: Intracellular Superoxide ($O_2^{\bullet-}$), D: Thiobarbituric Acid Reactive Substance (TBARS), E: Superoxide Dismutase (SOD) and F: Catalase. * $p < 0.05$

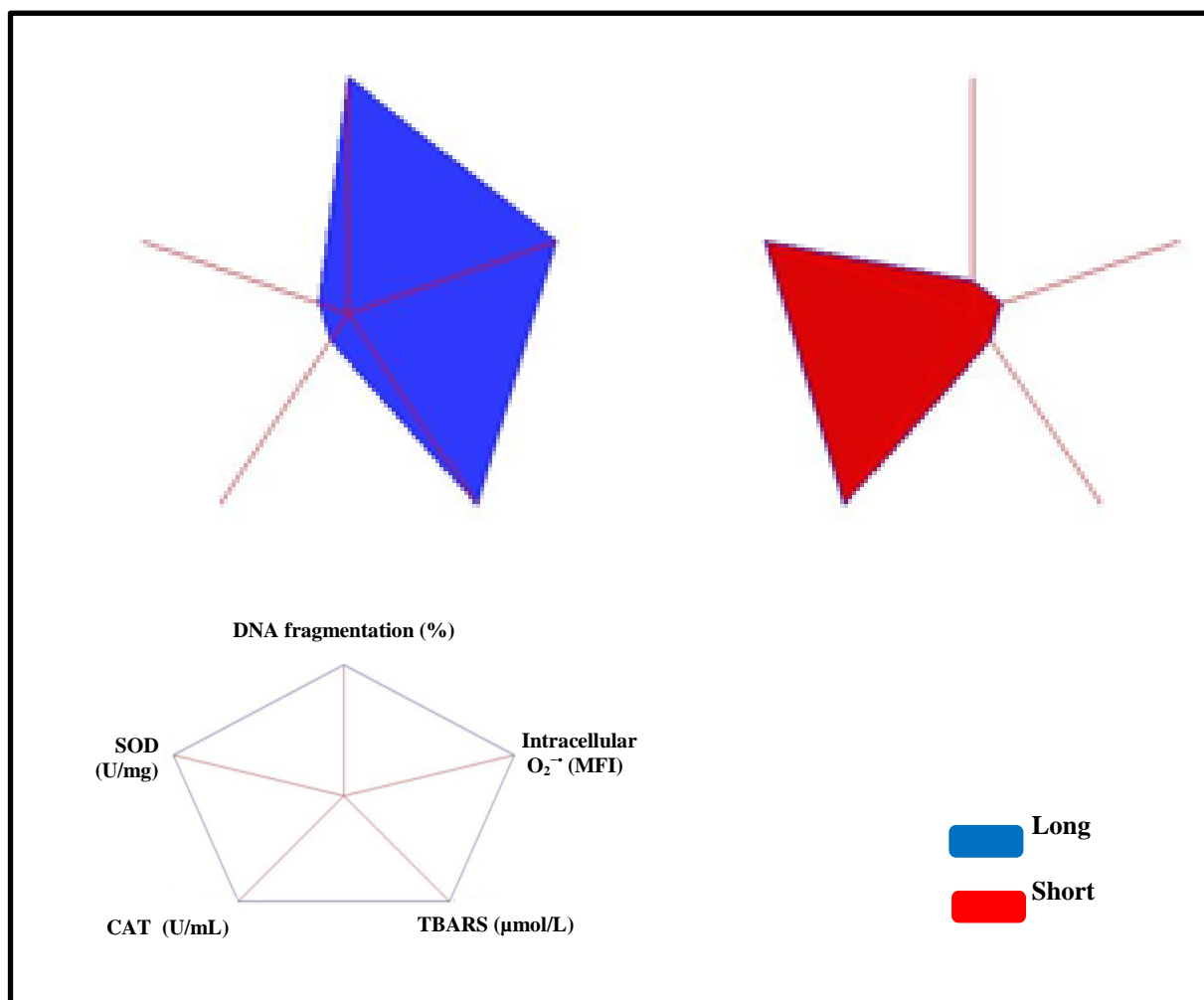


Figure 4. 9: Star symbol Plots comparing five advanced semen parameters between short and long periods of abstinence. O_2^- = superoxide anion, MFI = median DHE fluorescence intensity, TBARS = thiobarbituric acid reactive substances, SOD = superoxide dismutase. MFI = median florescent intensity, μmol = micromole, L = litre, U = unite, mg = milligram, mL = millilitre.

4.2 Aim II: Relationship between basic and advanced semen parameters.

The statistically analyzed data obtained from Aim II of the study, in which correlations between basic and advanced semen parameters were conducted, will be presented below. Results from ROC curve analysis for establishing cut-off values for advanced semen parameters from a number of basic semen parameters will be depicted in Figures 4.20-28.

4.2.1 Correlation between basic and advanced semen parameters

As depicted in Table 4.6, semen volume did not correlate significantly with any of the advanced parameters. Semen pH only correlated significantly with seminal TBARS and showed a negative correlation ($r = -0.47$; $p < 0.01$). Sperm concentration and T.S.C. did not correlate significantly with any of the advanced parameters. The percentage of viable spermatozoa correlated significantly and negatively with both intracellular $O_2^{\cdot-}$ levels ($r = -0.33$; $p = 0.04$) and sperm DNA fragmentation (-0.43 ; $p = 0.01$). The proportion of morphologically normal spermatozoa correlated significantly and negatively with the intracellular $O_2^{\cdot-}$ levels ($r = -0.39$; $p = 0.02$), and significantly and positively with seminal plasma CAT activity ($r = 0.33$; $p = 0.04$), while its correlations with other advanced parameters did not reach a statistical significance.

Table 4. 6: Correlation analysis between basic and advanced semen parameters.

		O₂⁻ (MFI)	TBARS (µmol/L)	CAT (U/mL)	SOD (U/mg)	Acrosome- intact (%)	DNA Fragmented (%)
		N = 40	N = 44	N = 44	N = 44	N = 40	N = 40
	Mean ± SEM	155.3±16.82	24.25±2.63	36.16±1.5	4.09±0.22	85.28±0.42	12.76±0.8
	Mean ± SD	155.3±106.4	24.25±17.48	36.16±10.0	4.09±1.44	85.28±2.68	12.76±5.04
	Median (25-75%)	124.5 (78.05-215.0)	18.65(9.5-41.15)	36.9(29.0-42.5)	3.9(3.0-5.03)	85.0(83.0-87.0)	12(11.2-15.7)
Volume (mL)	R	-0.05	0.03	-0.05	-0.15	-0.30	0.14
	p	0.75	0.83	0.76	0.32	0.06	0.39
	Mean ± SEM	2.89±0.21	2.68±0.21	2.68±0.21	68±0.21	2.485±0.20	2.61±0.21
	Mean ± SD	2.89±1.31	2.68±1.42	2.68±1.42	2.68±1.42	2.485±1.38	2.61±1.32
	Median (25-75%)	2.75(1.8-3.47)	2.2(1.73-3.2)	2.2(1.73-3.2)	2.2(1.73-3.2)	2.2(1.45-3.37)	2.35(1.62-2.8)
pH	R	-0.13	-0.47	-0.11	0.29	-0.04	-0.03
	p	0.43	<0.01	0.48	0.06	0.79	0.84
	Mean ± SEM	7.62±0.03	7.67±0.03	7.67±0.03	7.67±0.03	7.67±0.03	7.7±0.03
	Mean ± SD	7.62±0.17	7.67±0.2	7.67±0.2	7.67±0.2	7.67±0.2	7.7±0.19
	Median (25-75%)	7.5(7.5-7.7)	7.7(7.5-7.7)	7.7(7.5-7.7)	7.7(7.5-7.7)	7.7(7.5-7.7)	7.7(7.5-7.7)
Concentration (10⁶/mL)	R	0.10	-0.16	0.03	0.17	0.13	-0.24
	p	0.55	0.31	0.84	0.27	0.43	0.13
	Mean ± SEM	66.3±6.51	45.7±3.64	45.7±3.64	45.7±3.64	44.03±3.93	46.81±3.08
	Mean ± SD	66.3±41.23	45.7±22.96	45.7±22.96	45.7±22.96	44.03±24.84	46.81±19.48
	Median (25-75%)	51.95(36.35-96.45)	40.4(33.3-56.55)	40.4(33.3-56.5)	40.4(33.3-56.5)	41.45(27.7-56.73)	40.90(34.33-57.6)
T.S.C. (10⁶/ejaculate)	R	-0.01	-0.02	-0.02	-0.16	-0.14	0.03
	p	0.97	0.92	0.87	0.30	0.40	0.87
	Mean ± SEM	197.2±28.54	123.0±14.7	123.0±14.7	123.0±14.7	107.4±12.45	123.6±14.10
	Mean ± SD	197.2±180.5	123.0±97.24	123.0±97.24	123.0±97.24	107.4±78.73	123.6±89.21
	Median (25-75%)	144.8(80.28-251.1)	87.3(60.1-169.9)	87.3(60.1-169.9)	87.3(60.1-169.9)	91.3(51.73-132.9)	88.45(62.2-167.7)
Viability (%)	R	-0.33	0.02	0.01	0.03	0.15	-0.43
	p	0.04	0.91	0.95	0.86	0.36	0.01
	Mean ± SEM	68.79±1.34	67.5±1.43	67.5±1.43	67.5±1.43	70.33±1.14	67.53±1.3
	Mean ± SD	68.79±8.29	67.5±9.3	67.5±9.3	67.5±9.3	70.33±7.23	67.53±7.6
	Median (25-75%)	70(62.75-76.25)	68.0(62-75)	68.0(62-75)	68.0(62-75)	73.0(65.0-75.0)	67.50(62-73.5)
Normal morphology (%)	R	-0.39	-0.10	0.33	0.23	-0.30	0.08
	p	0.02	0.57	0.04	0.17	0.06	0.65
	Mean ± SEM	16.39±1.16	18.45±1.06	18.45±1.06	18.45±1.06	17.97±1.35	17.5±1.03
	Mean ± SD	16.39±6.95	18.45±6.57	18.45±6.57	18.45±6.57	17.97±7.64	17.5±6.0
	Median (25-75%)	16(10.5-25.7)	15.5(12.75-24.0)	15.5(12.7-24.0)	15.5(12.7-24.0)	17.5(12.5-23.5)	17.5(12.7-22.5)

O₂⁻ = superoxide anion, MFI = median DHE fluorescence intensity, TBARS = thiobarbituric acid reactive substances, CAT = catalase, SOD = superoxide dismutase. MFI = median fluorescent intensity, µmol = micromole, L = litre, U = unite, mg = milligram, mL = millilitre.

As shown in Table 4.7, the proportion of total motility only correlated significantly with seminal plasma CAT activity and showed a positive correlation ($r = 0.33$; $p = 0.03$). None of the motility and velocity variables listed in Table 4.7 correlated significantly with sperm intracellular $O_2^{\cdot-}$ levels, DNA fragmentation or seminal plasma SOD activity. The proportion of progressive motility correlated significantly and positively with seminal plasma CAT activity ($r = 0.31$; $p = 0.04$), and showed a marginally significant and negative correlation with seminal plasma TBARS levels ($r = -0.30$; $p = 0.05$). The proportion of rapidly motile spermatozoa correlated significantly and negatively with seminal TBARS levels ($r = -0.31$; $p = 0.04$), and significantly and positively with CAT activity ($r = 0.31$; $p = 0.04$), though its correlations with other advanced parameters did not reach a statistical significance. The proportion of spermatozoa with medium motility correlated significantly and positively with seminal TBARS levels ($r = 0.51$; $p < 0.01$), while slow spermatozoa correlated significantly with both seminal plasma TBARS levels ($r = 0.38$; $p = 0.01$) and the proportion of spermatozoa with acrosome-intact ($r = 0.41$; $p < 0.01$) and showed positive correlations. The proportion of static spermatozoa showed a significant and negative correlation with seminal plasma CAT activity ($r = -0.33$; $p = 0.03$), but its correlations with other advanced parameters were not significant. Principal Component Analysis representing the correlation between advanced semen parameters and basic sperm motility variables are illustrated in Figures 4.10, 11, 12, 13 and 14.

Table 4. 7: Correlation analysis between sperm motility/velocity and advanced semen parameters.

		O ₂ ⁻ (MFI)	TBARS (µmol/L)	CAT (U/mL)	SOD (U/mg)	Acrosome-intact (%)	DNA Fragmented (%)
		N=40	N=44	N=44	N=44	N=40	N=44
	Mean±SEM	155.3±16.82	24.25±2.63	36.16±1.5	4.09±0.22	4.09±0.22	12.76±0.8
	Mean±SD	155.3±106.4	24.25±17.48	36.16±10.0	4.09±1.44	4.09±1.44	12.76±5.04
	Median(25-75)	124.5(78.05-215.0)	18.65(9.53-41.15)	36.9(29.0-42.5)	3.9(3.0-5.03)	3.9(3.0-5.03)	12(11.2-15.7)
Motility (%)	R	-0.27	-0.09	0.33	0.05	0.14	-0.21
	p	0.09	0.56	0.03	0.75	0.37	0.19
	Mean ± SEM	65.4±2.71	61.6±1.94	61.6±1.94	61.6±1.94	66.85±2.43	59.09±2.24
	Mean ± SD	65.4±17.15	61.6±12.9	61.6±12.9	61.6±12.9	66.85±15.36	59.09±14.2
	Median(25-75%)	69.15(57.25-75.9)	63.7(58.53-69.65)	63.7(58.53-69.65)	63.7(58.53-69.65)	68.3(53.1-79.85)	62.55(52.9-69.3)
Progressive Motility (%)	R	-0.25	-0.30	0.31	0.13	0.09	-0.22
	p	0.12	0.05	0.04	0.41	0.58	0.17
	Mean ± SEM	52.0±2.6	47.82±1.85	47.82±1.85	47.82±1.85	51.58±2.43	45.67±2.02
	Mean ± SD	52.0±16.5	47.82±12.3	47.82±12.3	47.82±12.3	51.58±15.36	45.67±12.8
	Median(25-75%)	57.8(42.75-63.55)	48.95(41.7-56.5)	48.95(41.7-56.5)	48.95(41.7-56.5)	49.45(40.55-65.5)	47.4(39.73-54.95)
Rapid (%)	R	-0.26	-0.31	0.31	0.13	0.29	-0.22
	p	0.10	0.04	0.04	0.39	0.07	0.17
	Mean ± SEM	51.15±2.63	46.72±1.86	46.72±1.86	46.72±1.86	50.87±2.45	44.52±2.03
	Mean ± SD	51.15±16.7	46.72±12.33	46.72±12.33	46.72±12.33	50.87±15.47	44.52±12.9
	Median(25-75%)	57.05(42-63.4)	47.3(41.0-55.83)	47.3(41.0-55.83)	47.3(41.0-55.83)	48.55(39.33-78.2)	46.25(38.5-53.35)
Medium (%)	R	0.11	0.51	-0.15	-0.24	0.02	-0.03
	p	0.51	<0.01	0.32	0.12	0.88	0.84
	Mean ± SEM	6.24±0.35	6.6±0.33	6.6±0.33	6.6±0.33	5.49±0.33	6.3±0.39
	Mean ± SD	6.24±2.2	6.6±2.2	6.6±2.2	6.6±2.2	5.49±2.08	6.3±2.46
	Median(25-75%)	6.3(4.55-7.8)	6.25(5.02-8.5)	6.25(5.02-8.5)	6.25(5.02-8.5)	5.40(4.15-6.93)	6.05(3.9-5.6)
Slow (%)	R	0.06	0.38	-0.05	-0.07	0.41	0.10
	p	0.72	0.01	0.73	0.67	<0.01	0.54
	Mean ± SEM	8.0±0.35	8.25±0.4	8.25±0.4	8.25±0.4	10.43±0.47	8.22±
	Mean ± SD	8.0±2.23	8.25±2.5	8.25±2.5	8.25±2.5	10.43±10.5	8.22±0.44
	Median(25-75%)	7.7(6.65-9.25)	8.35(6.7-9.52)	8.35(6.7-9.52)	8.35(6.7-9.52)	10.5(8.27-12.08)	7.7(6.52-9.52)
Static (%)	R	0.27	0.09	-0.33	-0.05	-0.13	0.20
	p	0.09	0.57	0.03	0.77	0.41	0.21
	Mean ± SEM	34.61±2.71	38.45±1.94	38.45±1.94	38.45±1.94	33±2.47	41.0±2.23
	Mean ± SD	34.61±17.13	38.45±12.9	38.45±12.9	38.45±12.9	33±15.6	41.0±14.11
	Median(25-75%)	30.9(24.13-42.68)	36.3(30.35-41.5)	36.3(30.35-41.5)	36.3(30.35-41.5)	31.75(20.15-46.85)	37.45(30.8-47.13)

O₂⁻ = superoxide anion, TBARS = thiobarbituric acid reactive substances, CAT = catalase, SOD = superoxide dismutase, MFI = median DHE fluorescence intensity, µmol = micromole, L = litre, U = unite, mg = milligram, mL = millilitre.

The correlation between each advanced variable (arrows with blue lettering) and basic semen variables (arrows with red lettering) are depicted in the following correlation circles of the Principal Component Analysis (Figures 4.10-19). In each analysis, an advanced variable (i.e. SOD) is compared to several basic semen parameters. As previously mentioned (3.4.1), arrows pointing roughly in the same direction to the relevant advanced variable have a positive correlation with the variable, while arrows pointing in the opposite direction are negatively correlated. Similarly, arrows perpendicular to the direction of the advanced variable indicates no correlation. Ideally, the arrows should stretch into the outer circle to be considered significant.

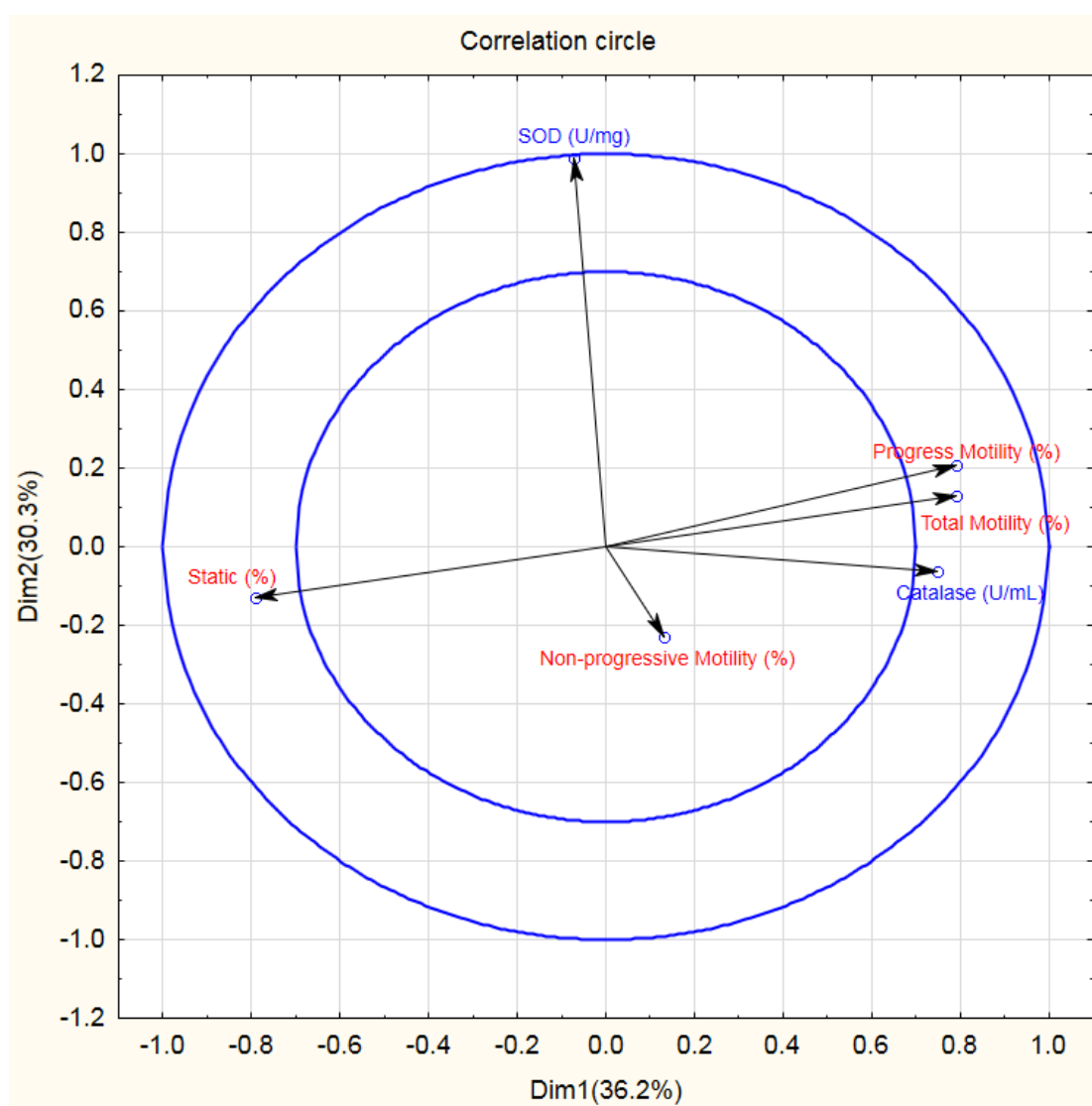


Figure 4. 10: Correlation circle of the Principal Component Analysis. Correlations of seminal plasma catalase (U/mL) and SOD (U/mg) activity with basic motility parameters. SOD = superoxide dismutase, U = unite, mg = milligram, mL = millilitre.

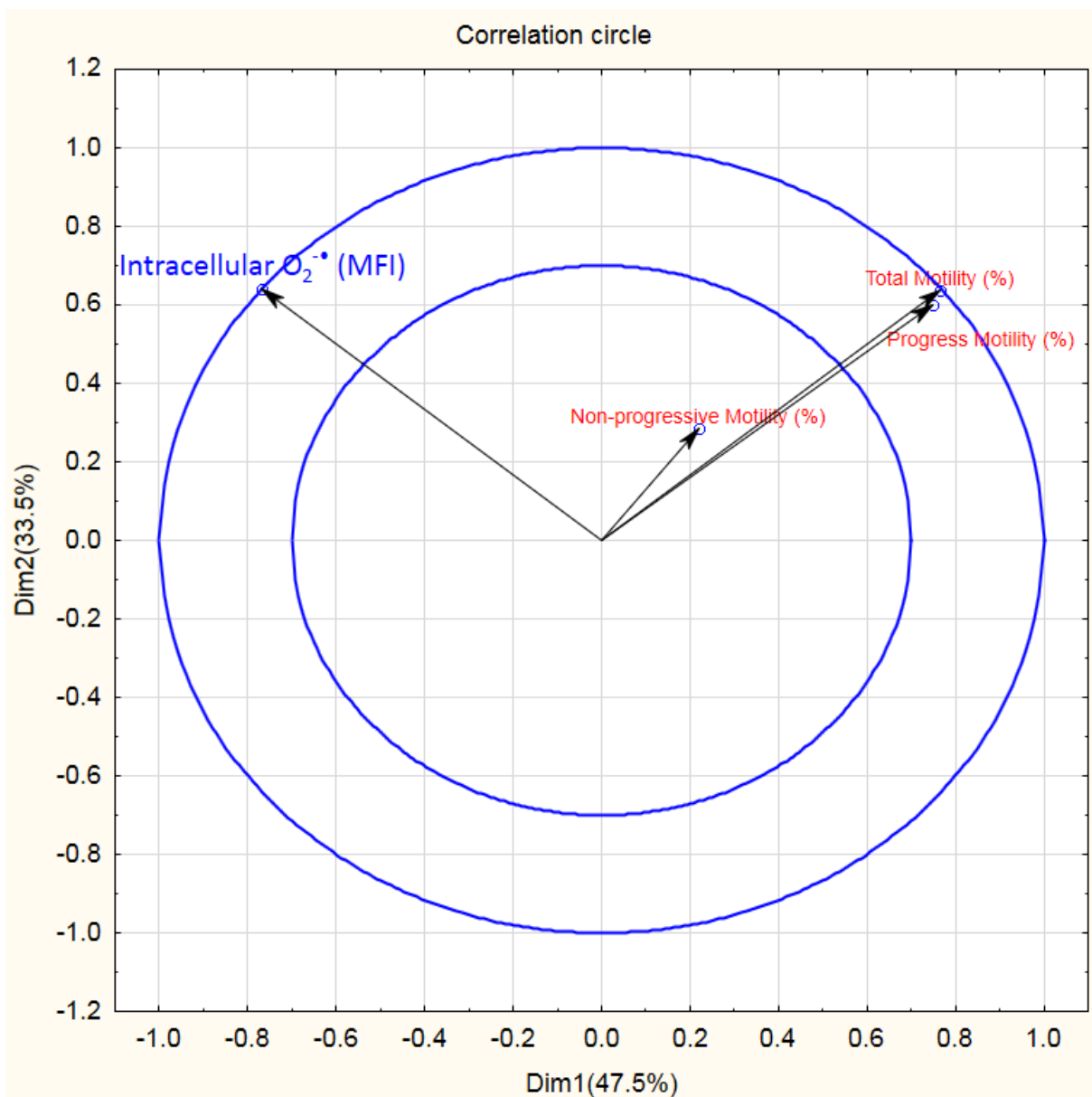


Figure 4. 11: Correlation circle of the Principal Component Analysis. Correlations of intracellular O₂^{-•} with basic motility parameters. O₂^{-•} = superoxide anion, MFI = median fluorescence intensity

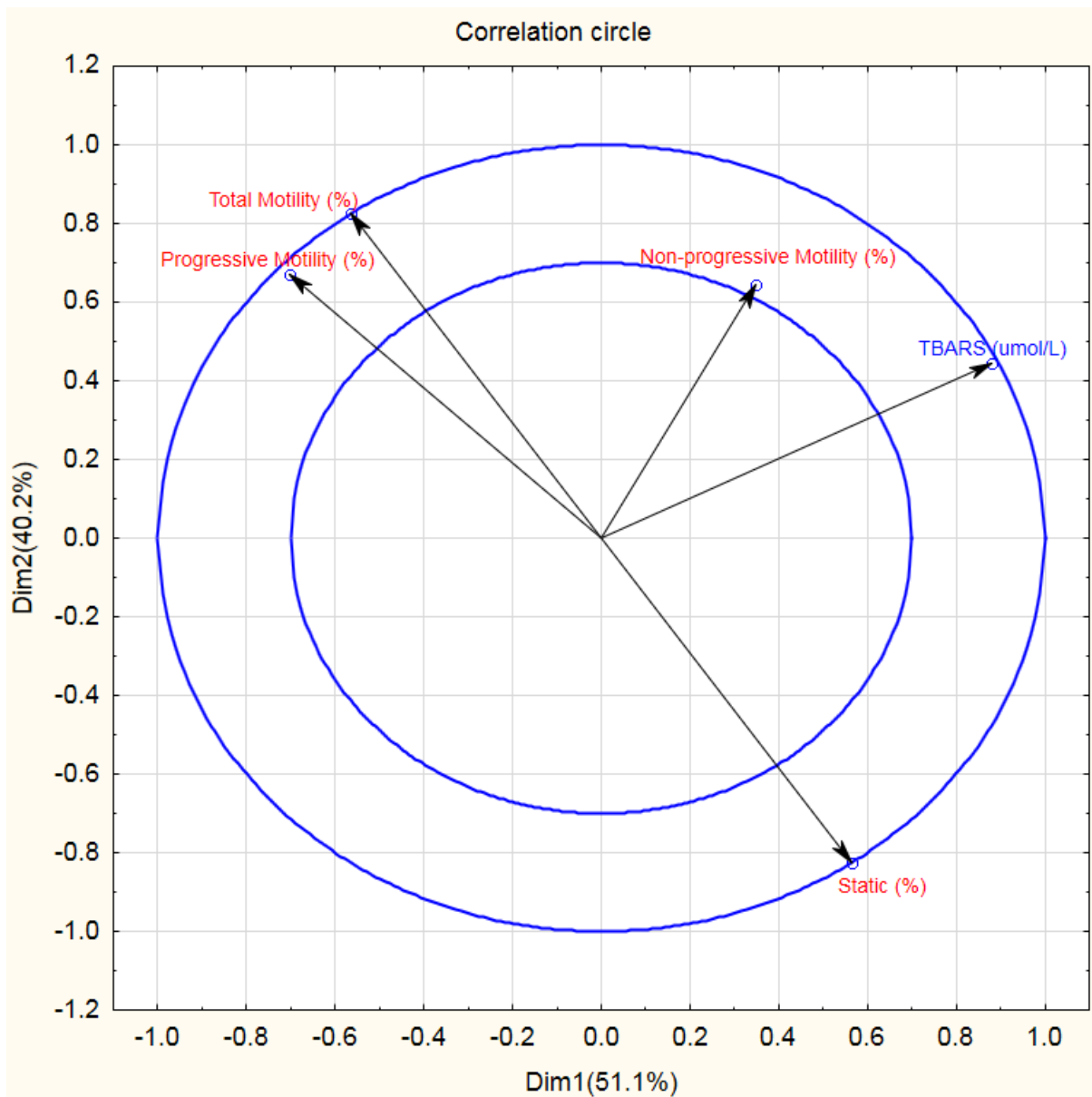


Figure 4. 12: Correlation circle of the Principal Component Analysis. Correlations of seminal plasma TBARS levels with basic motility parameters. TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre.

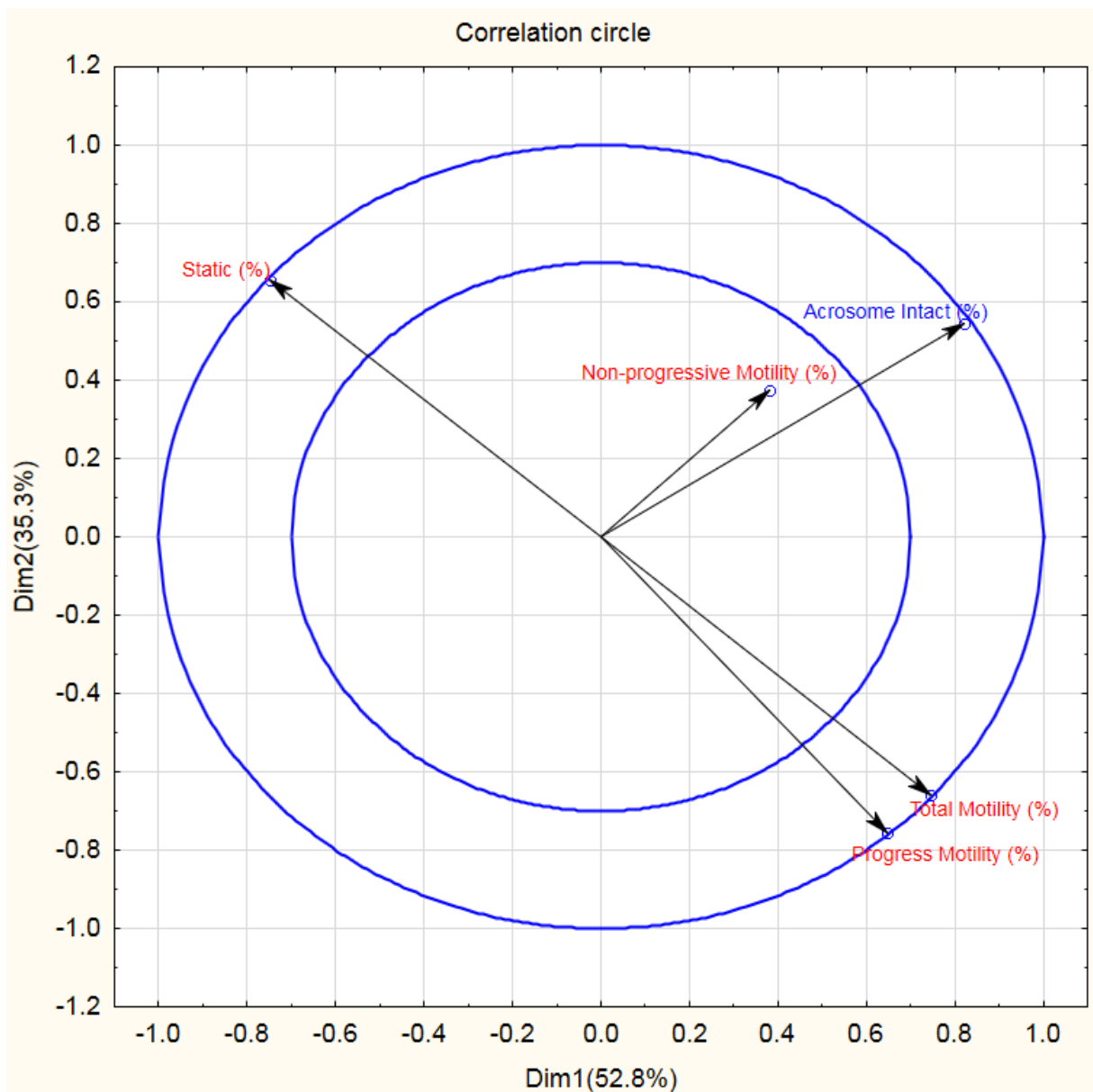


Figure 4. 13: Correlation circle of the Principal Component Analysis. Correlations of sperm acrosome-intact with basic motility parameters.

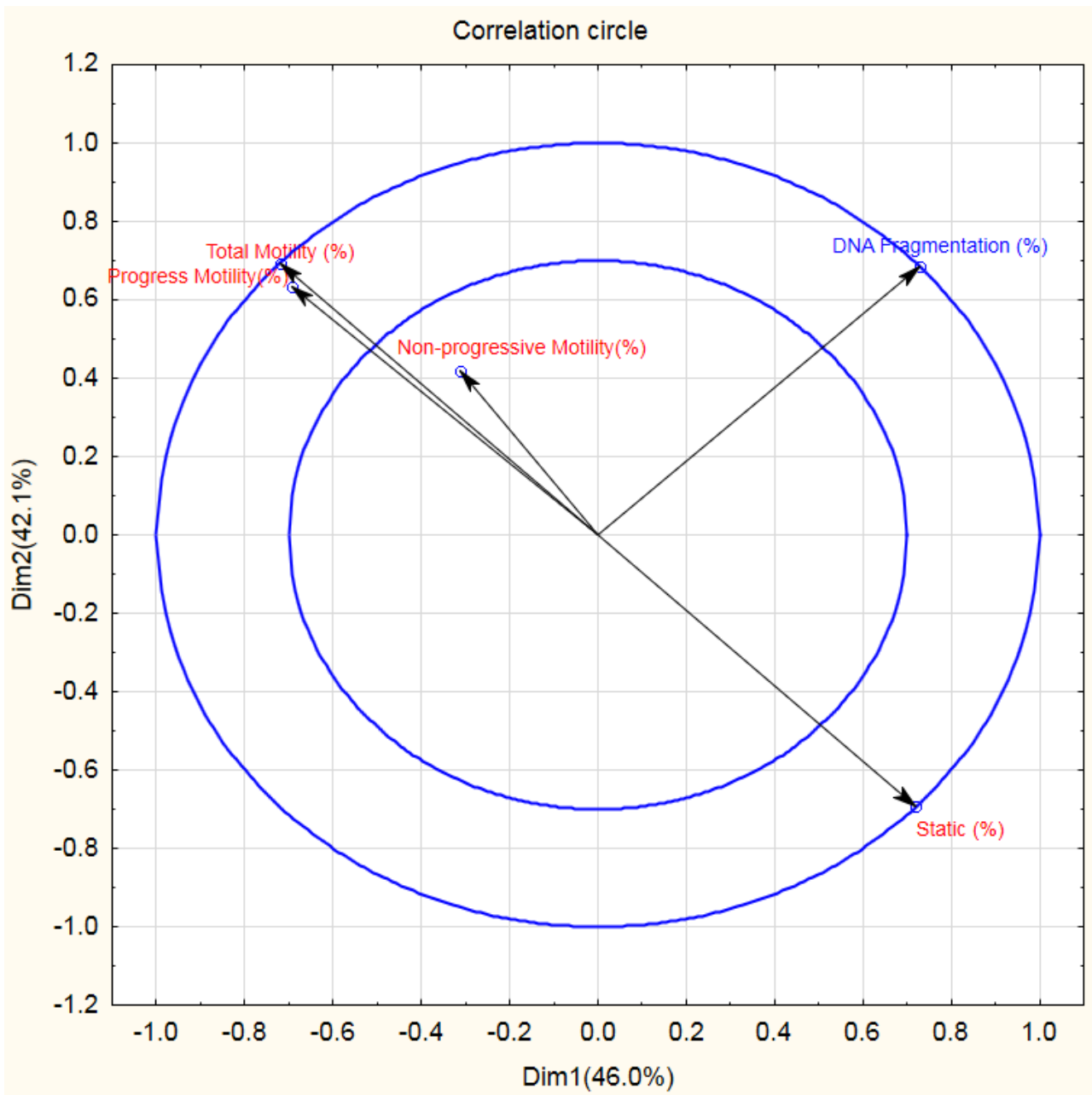


Figure 4. 14: Correlation circle of the Principal Component Analysis. Correlations of sperm DNA fragmentation with basic motility parameters.

As shown in Table 4.8, VCL was correlated significantly and negatively with both intracellular $O_2^{\cdot-}$ ($r = -0.46$; $p < 0.01$), and seminal plasma TBARS levels ($r = -0.62$; $p < 0.01$), while its correlation with seminal SOD levels was significant and positive ($r = 0.35$; $p = 0.02$). VSL was correlated significantly and negatively with the seminal TBAR levels ($r = -0.33$; $p = 0.03$) and significantly and positively with the percentage of DNA fragmentation ($r = 0.36$; $p = 0.02$). The correlation between VAP and both intracellular $O_2^{\cdot-}$ ($r = -0.43$; $p < 0.01$), and seminal plasma TBARS levels ($r = -0.60$; $p < 0.01$), was significant and negative, while its correlation with other advanced parameters was not statistically significant. Both LIN ($r = 0.48$; $p < 0.01$) and STR ($r = 0.43$; $p < 0.01$) were significantly and positively correlated with the proportion of DNA fragmented spermatozoa. ALH was correlated significantly and negatively with intracellular $O_2^{\cdot-}$ ($r = -0.49$; $p < 0.01$), and DNA fragmentation ($r = -0.42$; $p < 0.01$), while its correlation with the seminal plasma SOD activity was significant and positive ($r = 0.33$; $p = 0.03$). A significant negative correlation was shown between BCF and seminal plasma TBARS levels ($r = -0.56$; $p < 0.01$). The correlation between advanced semen parameters and sperm kinematics are depicted in Figures 4.15, 16, 17, 18 and 19.

Table 4. 8: Correlation analysis between sperm kinematics and advanced semen parameters.

	O₂⁻ (MFI)	O₂⁻ (MFI)	TBARS (µmol/L)	CAT (U/mL)	SOD (U/mg)	Acrosome-intact (%)	DNA Fragmented (%)
	N=	N=40	N=44	N=44	N=44	N=40	N=44
	Mean±SEM	155.3±16.82	24.25±2.63	36.16±1.5	4.09±0.22	4.09±0.22	12.76±0.8
	Mean±DS	155.3±106.4	24.25±17.48	36.16±10.0	4.09±1.44	4.09±1.44	12.76±5.04
	Median (25-75)	124.5 (78.05-215.0)	18.65(9.53-41.15)	36.9(29.0-42.5)	3.9(3.0-5.03)	3.9(3.0-5.03)	12(11.2-15.7)
VCL	R	-0.46	-0.62	0.14	0.35	-0.12	-0.09
(µm/s)	p	<0.01	<0.01	0.36	0.02	0.46	0.60
	Mean ± SEM	83.33±2.35	77.9±1.82	77.9±1.82	77.9±1.82	83.52±3.15	77.05±2.0
	Mean ± SD	83.33±14.85	77.9±12.1	77.9±12.1	77.9±12.1	83.52±19.93	77.05±12.4
	Median (25-75%)	81.65(74.43-88.85)	75.1(69.6-83.9)	75.1(69.6-83.9)	75.1(69.6-83.9)	83.52(71.20-89.43)	74.65(68.75-82.25)
VSL	R	-0.18	-0.33	0.00	-0.10	0.12	0.36
(µm/s)	p	0.26	0.03	0.99	0.51	0.47	0.02
	Mean ± SEM	32.53±1.04	30.73±0.7	30.73±0.7	30.73±0.7	31.9±0.91	30.75±0.81
	Mean ± SD	32.53±6.55	30.73±4.45	30.73±4.45	30.73±4.45	31.9±5.77	30.75±5.1
	Median (25-75%)	31.05(27.7-35.7)	29.95(27.83-33.7)	29.95(27.83-33.7)	29.95(27.83-33.7)	31.8(28.48-35.50)	29.65(27.7-33.8)
VAP	R	-0.43	-0.60	0.06	0.25	-0.08	0.15
(µm/s)	p	<0.01	<0.01	0.71	0.11	0.61	0.36
	Mean ± SEM	53.15±1.3	51.74±0.9	51.74±0.9	51.74±0.9	52.83±1.51	51.4±1.0
	Mean ± SD	53.15±8.22	51.74±5.95	51.74±5.95	51.74±5.95	52.83±9.59	51.4±6.31
	Median (25-75%)	52.15(48.53-56.4)	50.7(48.28-55.45)	50.7(48.28-55.45)	50.7(48.28-55.45)	51.25(46.98-56.33)	50.7(47.93-55.2)
LIN	R	0.24	0.01	-0.11	-0.30	0.28	0.48
(%)	p	0.14	0.97	0.46	0.05	0.09	<0.01
	Mean ± SEM	39.52±1.12	40.03±1.0	40.03±1.0	40.03±1.0	39.13±1.06	40.5±1.13
	Mean ± SD	39.52±7.02	40.03±6.61	40.03±6.61	40.03±6.61	39.13±6.74	40.5±1.14
	Median (25-75%)	38.3(35.83-44.73)	40.8(34.03-44.4)	40.8(34.03-44.4)	40.8(34.03-44.4)	40.55(35.9-44.39)	40.8(35.0-44.53)
STR	R	0.14	0.03	0.01	-0.25	0.24	0.43
(%)	p	0.38	0.83	0.97	0.10	0.14	<0.01
	Mean ± SEM	61.29±1.3	59.6±1.04	59.6±1.04	59.6±1.04	60.84±1.19	60.1±1.21
	Mean ± SD	61.29±8.2	59.6±6.9	59.6±6.9	59.6±6.9	60.84±7.5	60.1±7.7
	Median (25-75%)	61.2(56.03-68.0)	59.4(54.03-65.0)	59.4(54.03-65.0)	59.4(54.03-65.0)	61.45(56.55-67.2)	59.4(54.5-65.85)
ALH	R	-0.49	-0.18	0.09	0.33	-0.26	-0.42
(µm)	p	<0.01	0.25	0.56	0.03	0.11	<0.01
	Mean ± SEM	2.1±0.07	1.9±0.04	1.9±0.04	1.9±0.04	2.23±0.08	1.85±0.04
	Mean ± SD	2.1±0.42	1.9±0.25	1.9±0.25	1.9±0.25	2.23±0.54	1.85±0.23
	Median (25-75%)	2.0(1.8-2.2)	1.8(1.7-2.0)	1.8(1.7-2.0)	1.8(1.7-2.0)	2.05(1.82-2.47)	1.8(1.7-1.9)
BCF	R	-0.03	-0.56	0.26	0.16	-0.08	0.20
(Hz)	p	0.84	<0.01	0.09	0.30	0.64	0.22
	Mean ± SEM	14.7±0.4	13.7±0.34	13.7±0.34	13.7±0.34	14.19±0.39	13.71±0.38
	Mean ± SD	14.7±2.52	13.7±2.29	13.7±2.29	13.7±2.29	14.19±2.51	13.71±2.43
	Median (25-75%)	14.95(12.7-16.45)	13.7(12.3-15.3)	13.7(12.3-15.3)	13.7(12.3-15.3)	15.2(12.98-15.7)	14.0(12.23-15.3)

O₂⁻ = superoxide anion, MFI = median DHE fluorescence intensity, TBARS = thiobarbituric acid reactive substances, CAT = catalase, SOD = superoxide dismutase, MFI = median fluorescence intensity µmol = micromole, L = litre, U = unite, mg = milligram, mL = millilitre, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, µm = micrometre, s = second, Hz = hertz

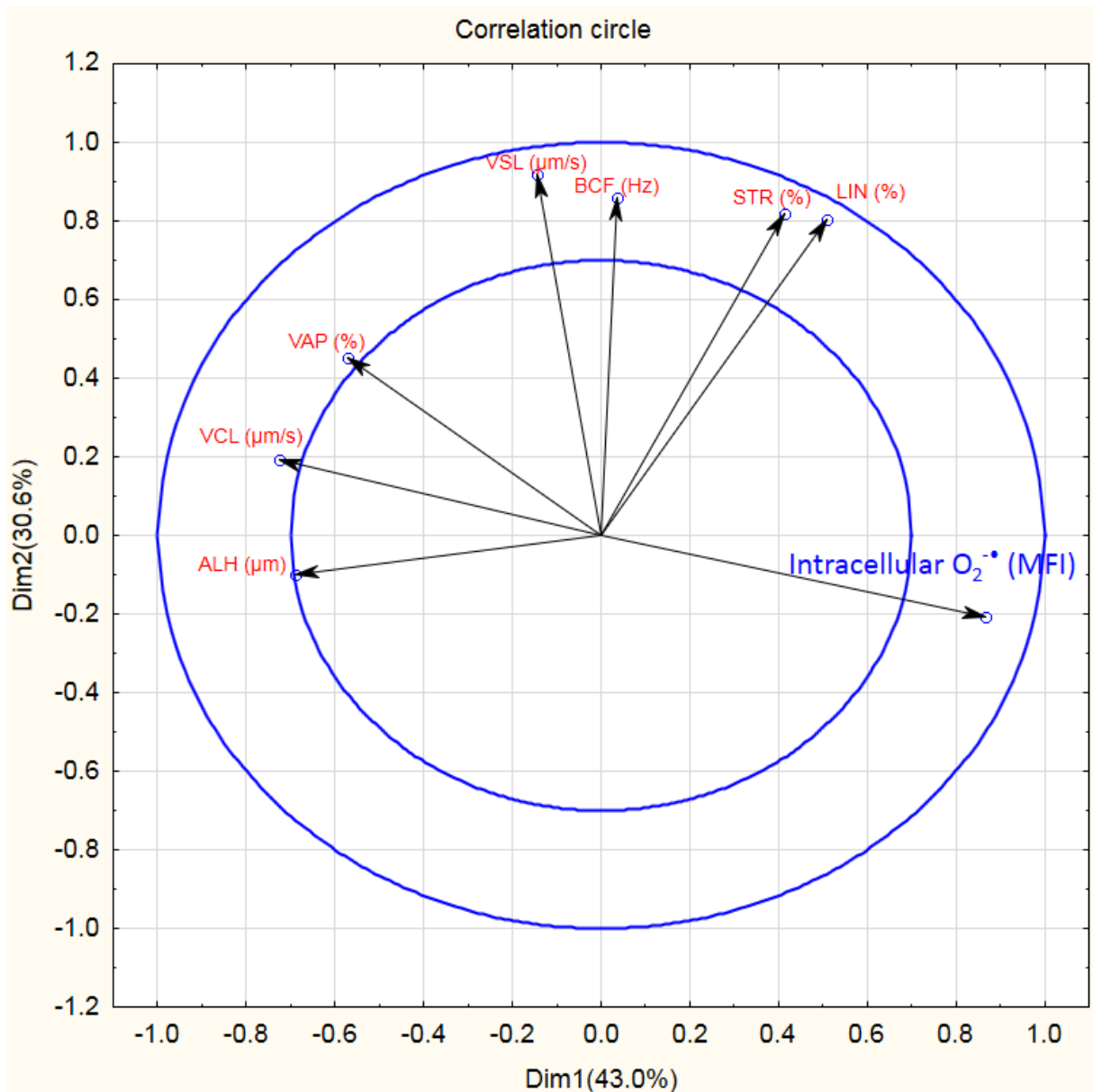


Figure 4. 15: Correlation circle of the Principal Component Analysis. Correlations of sperm intracellular $\text{O}_2^{\bullet-}$ with kinematic parameters. $\text{O}_2^{\bullet-}$ = superoxide anion, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, MFI = median fluorescence intensity, μm = micrometre, s = second, Hz = hertz

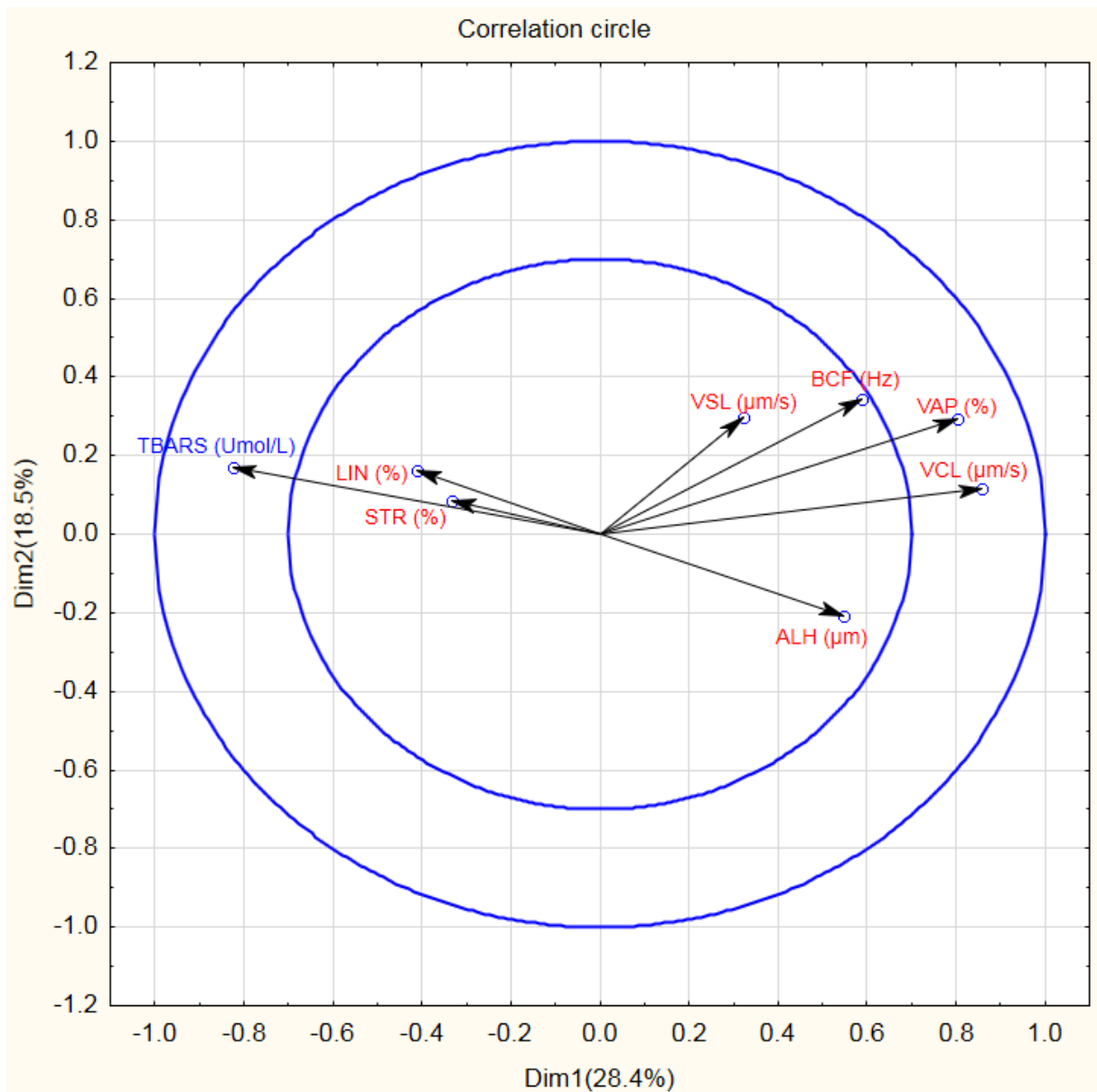


Figure 4. 16: Correlation circle of the Principal Component Analysis. Correlations of seminal plasma TBARS with sperm kinematic parameters. VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, μmol = micromole, L = litre, μm = micrometre, s = second, Hz = hertz.

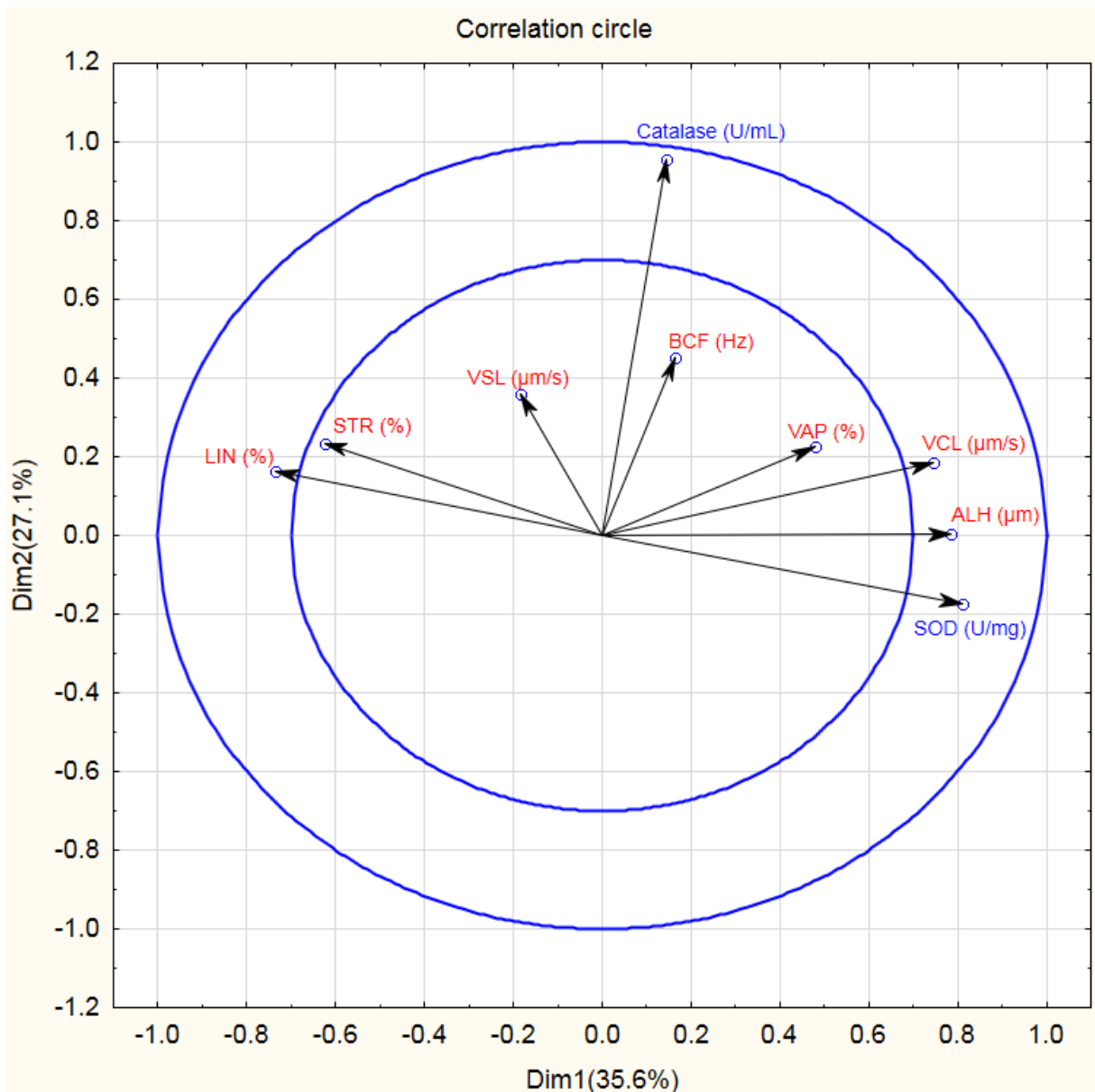


Figure 4. 17: Correlation circle of the Principal Component Analysis. Correlations of seminal plasma catalase and SOD activity with sperm kinematic parameters. SOD = superoxide dismutase, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, U = unite, mL = millilitre, mg = milligram, μm = micrometre, s = second, Hz = hertz.

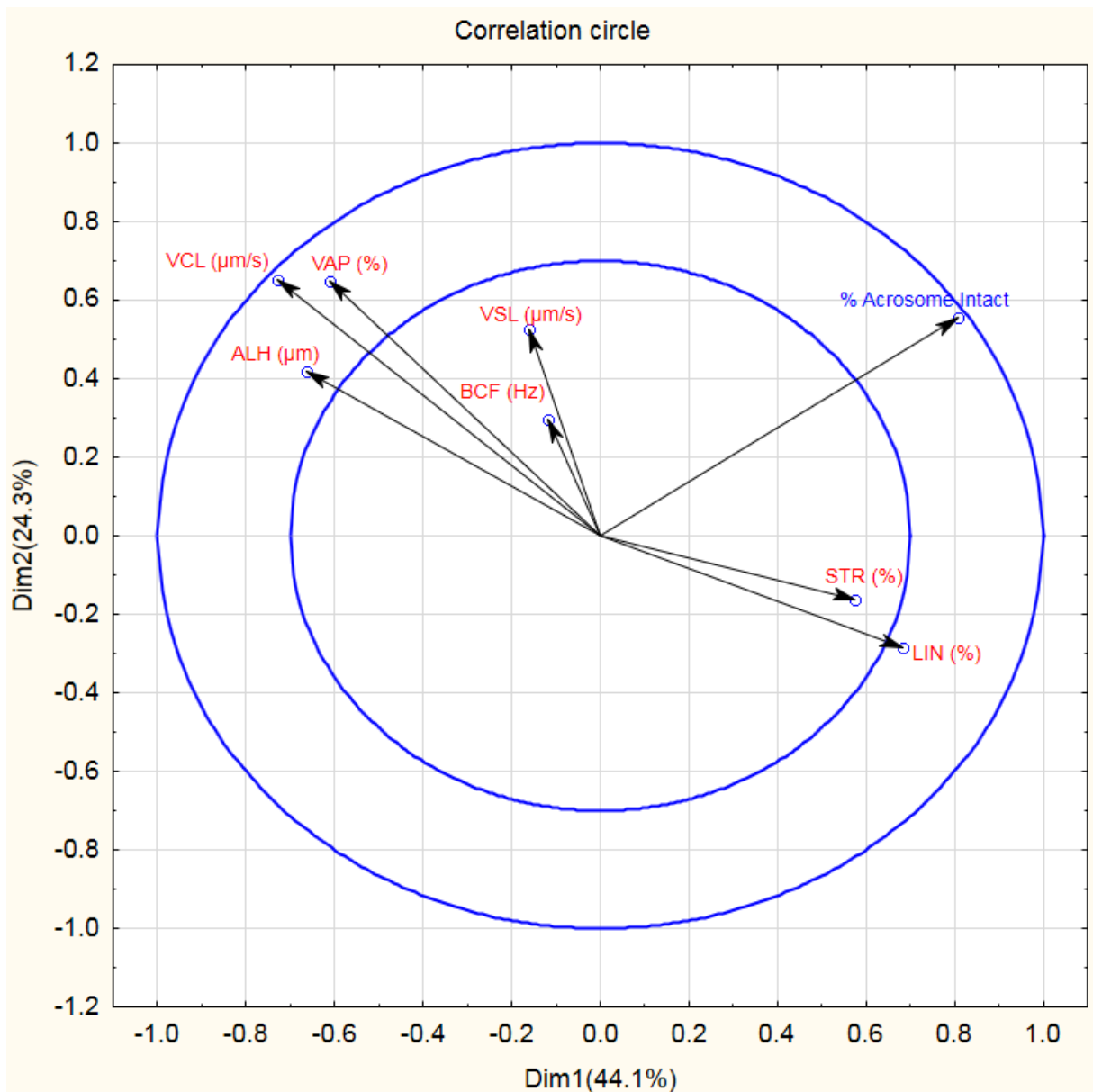


Figure 4. 18: Correlation circle of the Principal Component Analysis. Correlations of sperm acrosome-intact with kinematic parameters. VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, μm = micrometre, s = second, Hz = hertz.

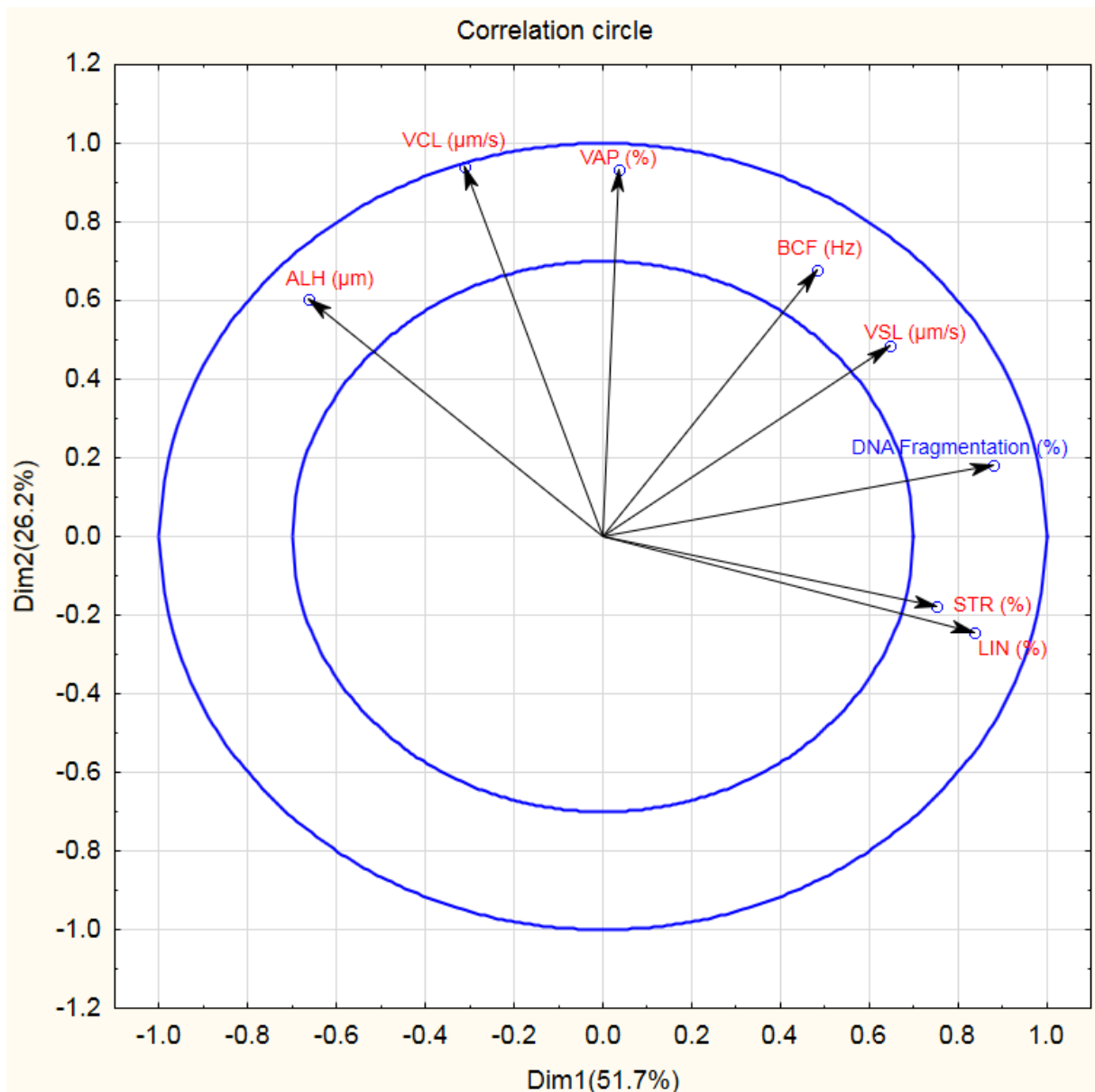


Figure 4. 19: Correlation circle of the Principal Component Analysis. Correlations of sperm DNA fragmentation with kinematic parameters. VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency, U = unite, mL = millilitre, mg = milligram, μm = micrometre, s = second, Hz = hertz.

4.2.2 Establishing cut-off values for the advanced semen parameters from basic semen parameters

The advanced semen parameters were stratified into two populations according to the lower reference limit of various basic semen parameters as supplied by the WHO (2010). Positive and negative predicted values as well as sensitivity and specificity were calculated using ROC curve analyses. After conducting ROC analysis, it came to light that the WHO lower reference value of 40 % for sperm total motility was associated with 227 MFI as a cut-off value for intracellular $O_2^{\cdot-}$ ($p < 0.01$), with a sensitivity and specificity of 80 % and 86 % respectively and an AUC of 0.83 as shown in Figure 4.20 A. The distribution of the advanced semen parameter values in samples with normal and below basic semen WHO reference values is shown in Figures 4.20 B–28 B.

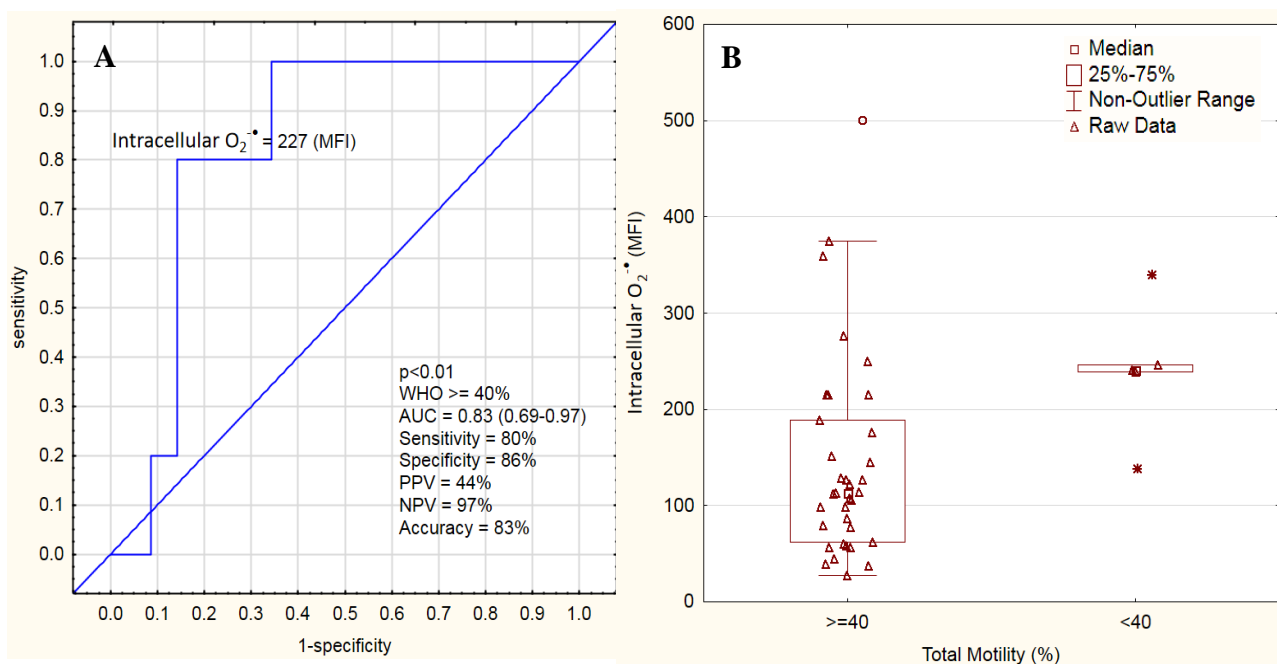


Figure 4.20: A. Receiver operator characteristic (ROC) curve showing $O_2^{\cdot-}$ (MFI) cut-off value and the area under the curve (AUC). B. Distribution of $O_2^{\cdot-}$ values in samples with normal and below WHO total motility reference values. $O_2^{\cdot-}$ = superoxide, MFI = median fluorescence intensity, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. ≥ 40 , 35 samples, < 40 ; 5 samples.

At the WHO minimum reference value of 32 % for progressive motility, the cut-off value for intracellular $O_2^{\cdot-}$ was 128 MFI ($p=0.01$), with a 100 % sensitivity and 65 % specificity and 0.79 as AUC (Figure 4.21 A), while the cut-off value for seminal plasma CAT activity was 37.3 U/mL ($p = 0.07$), with sensitivity and specificity of 100 % and 59 % respectively and 0.70 as AUC (Figure 4.22 A).

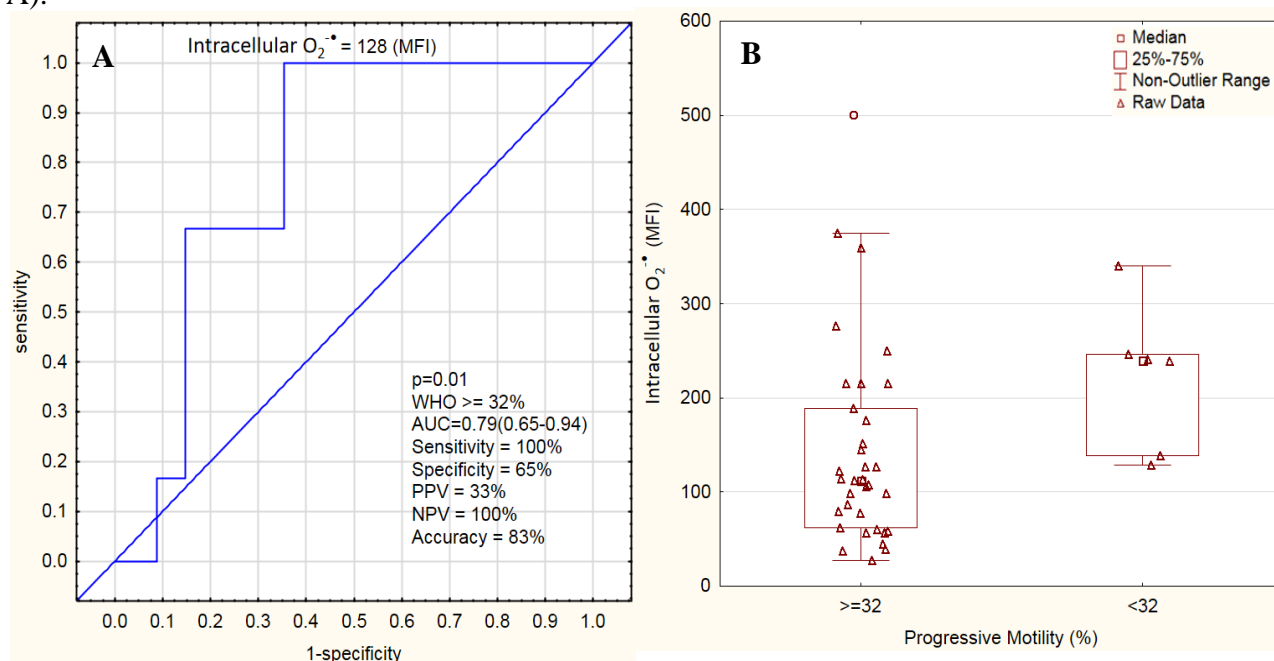


Figure 4. 21: A. Receiver operator characteristic (ROC) curve showing $O_2^{\cdot-}$ (MFI) cut-off value and the area under the curve. B. Distribution of $O_2^{\cdot-}$ values in samples with normal and below WHO progressive motility reference values. $O_2^{\cdot-}$ = superoxide, MFI = median fluorescence intensity, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. ≥ 32 , $n=33$; <32 , $n=7$.

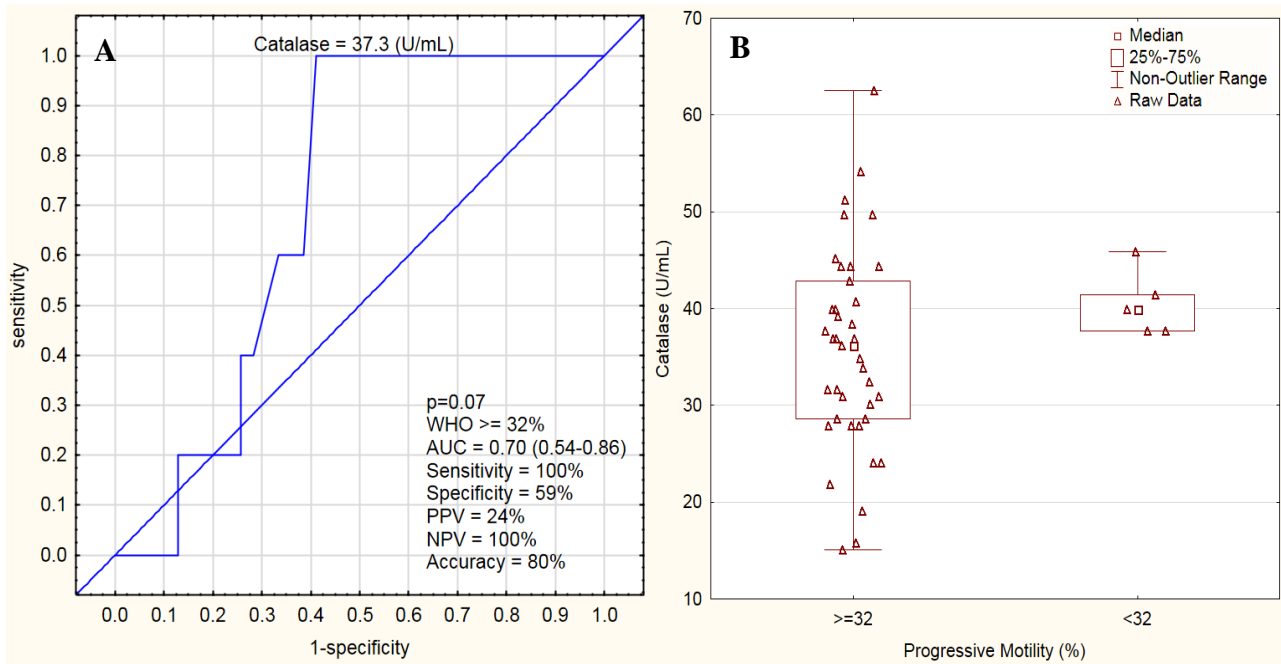


Figure 4.22: A. Receiver operator characteristic (ROC) curve showing seminal catalase activity (U/mL) cut-off value and the area under the curve. B. Distribution of catalase activity values in samples with normal and below WHO progressive motility reference values. WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value, U = unit, mL = millilitre. \geq 32, 38 samples, $<$ 32; 6 samples.

The lower WHO progressive motility reference value was also associated with the cut-off value of 17.7 $\mu\text{mol/L}$ ($p = 0.07$) for TBARS, with 80 % sensitivity, 51 % specificity and an AUC of 0.71 (Figure 4.23 A). At the same lower progressive motility reference value, the cut-off value for SOD activity was 3.445 U/mg ($p = 0.49$), with a sensitivity and specificity of only 56 % and 60 % respectively and an AUC of 0.51 (Figure 4.24 A). Similarly, the cut-off value for DNA fragmentation was 15.6 % ($p = 0.42$), with sensitivity and specificity of 80 % and 60 % respectively and 0.53 as AUC (Figure 4.25 A).

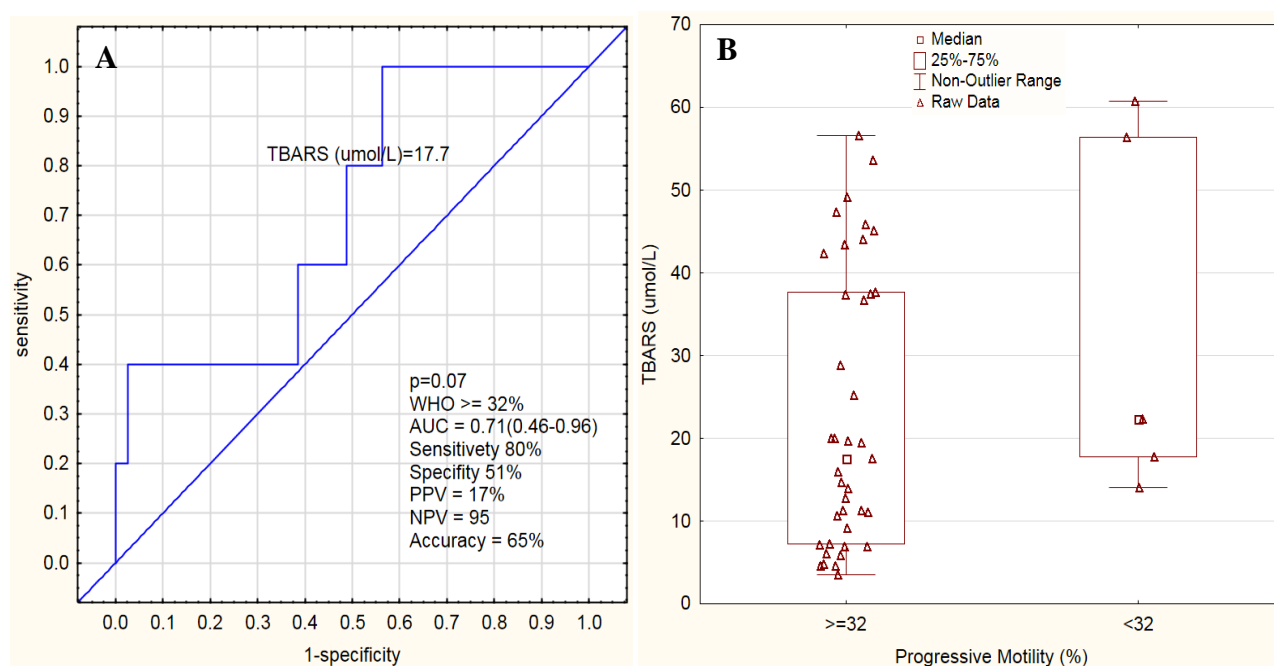


Figure 4. 23: A. Receiver operator characteristic (ROC) curve showing TBARS (Umol/L) cut-off value and the area under the curve. B. Distribution of TBARS values in samples with normal and below WHO progressive motility reference values. TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. ≥ 32 , 38 samples, < 32 ; 6 samples.

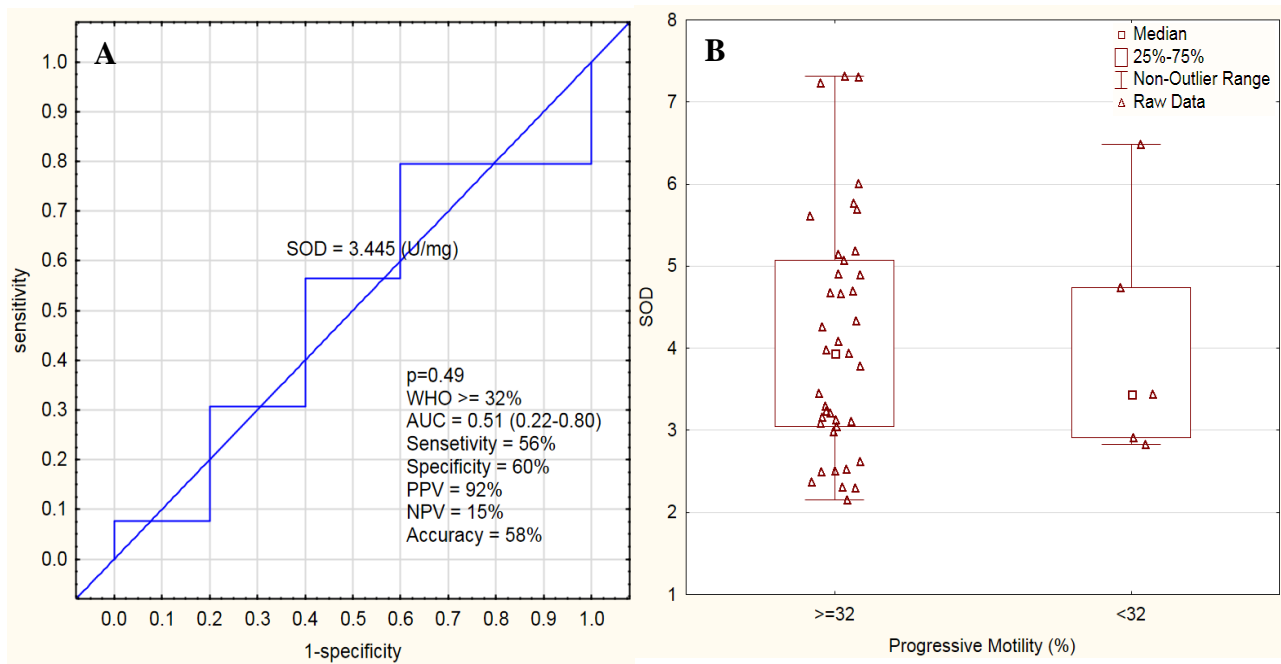


Figure 4. 24: A. Receiver operator characteristic (ROC) curve showing seminal SOD activity (U/mg) cut-off value and the area under the curve. B. Distribution of SOD activity values in samples with normal and below WHO progressive motility reference values. SOD =superoxide dismutase, U = unit, mg = milligram, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. \geq 32, 38 samples, <32; 6 samples.

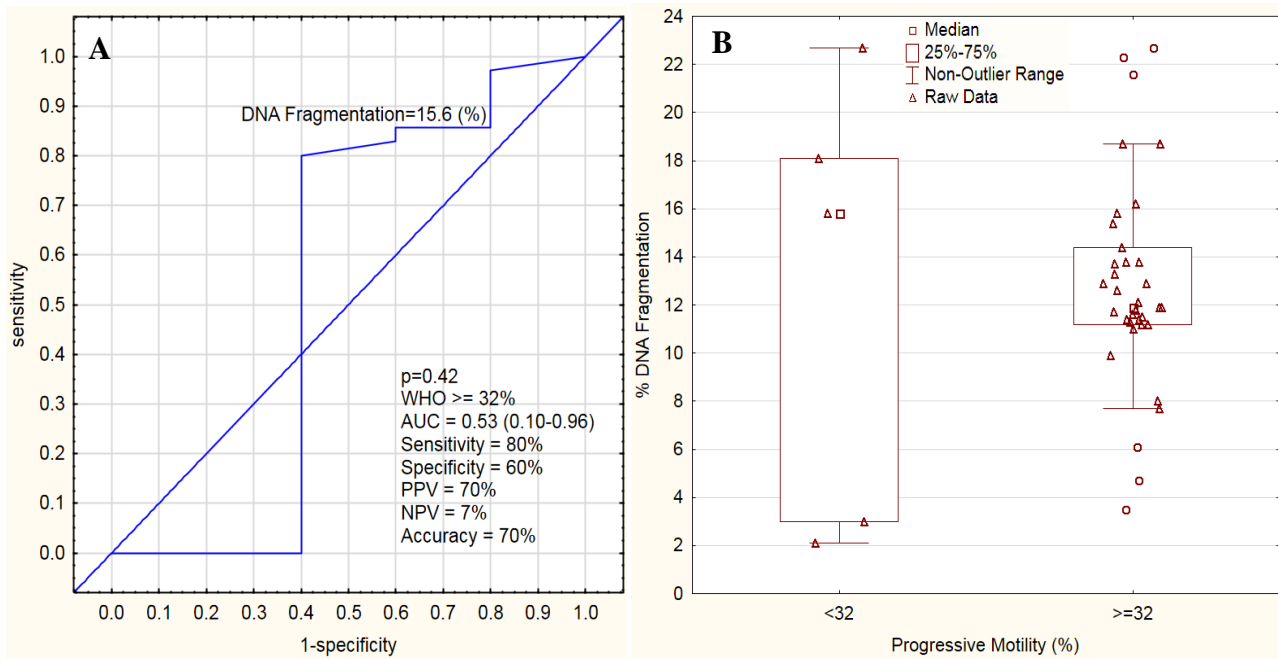


Figure 4. 25: A. Receiver operator characteristic (ROC) curve showing DNA fragmentation (%) cut-off value and the area under the curve. B. Distribution of DNA fragmentation percentages in samples with normal and below WHO progressive motility reference values. WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. \geq 32, 38 samples, <32; 6 samples.

The WHO minimum reference value of 58 % for sperm viability was associated with seminal plasma TBARS cut-off value of 9.86 $\mu\text{mol/L}$ ($p = 0.02$) with sensitivity and specificity of 81 % and 80 % respectively and an AUC of 0.79 (Figure 4.26 A). At the same sperm viability reference value, CAT activity cut-off value was 37.3 U/mL ($p = 0.07$) with a 100 % sensitivity and 59 % specificity and 0.70 as AUC (Figure 4.27 A), while the cut-off value for SOD activity was 3.22 U/mg ($p = 0.52$) with sensitivity and specificity of 60 % and 65 % respectively, and an AUC of only 0.50 (Figure 4.28 A).

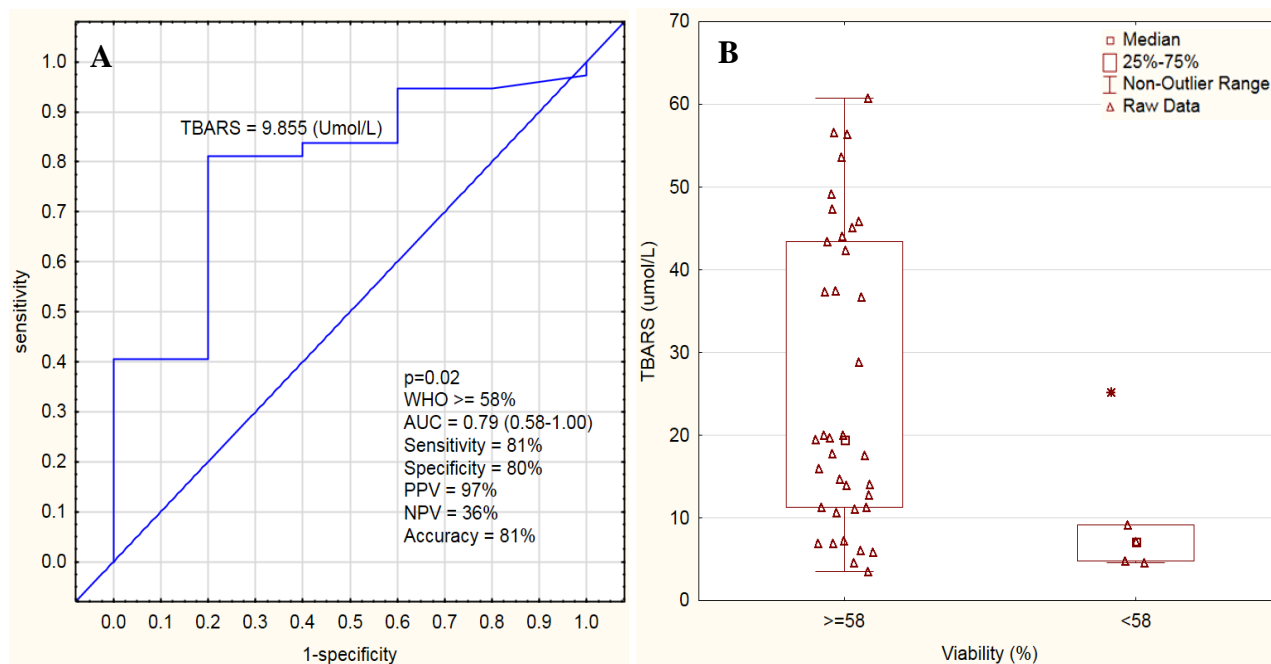


Figure 4. 26: A. Receiver operator characteristic (ROC) curve showing TBARS ($\mu\text{mol/L}$) cut-off value and the area under the curve (AUC). B. Distribution of TBARS values in samples with normal and below WHO viability reference values TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. ≥ 58 , 38 samples, < 58 ; 6 samples.

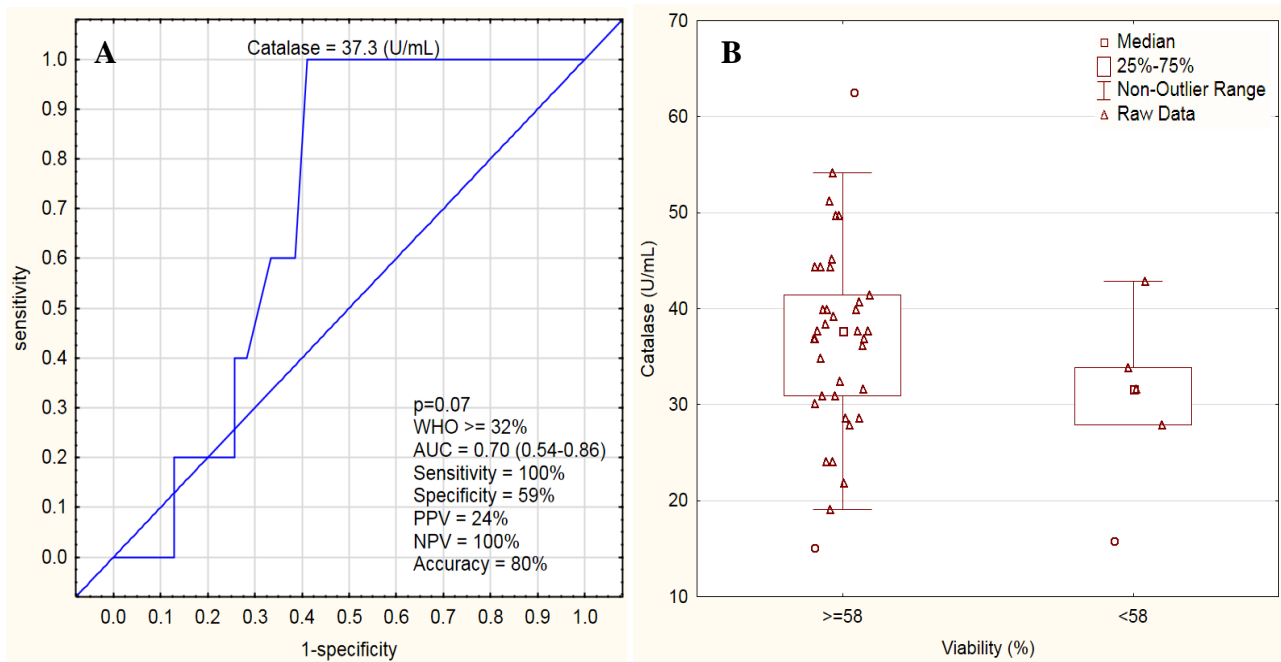


Figure 4.27: A. Receiver operator characteristic (ROC) curve showing seminal catalase activity (U/mL) cut-off value and the area under the curve. B. Distribution of catalase activity values in samples with normal and below WHO sperm viability reference values. WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value, U = unit, mL = millilitre. ≥ 58 , 38 samples, < 58 ; 6 samples.

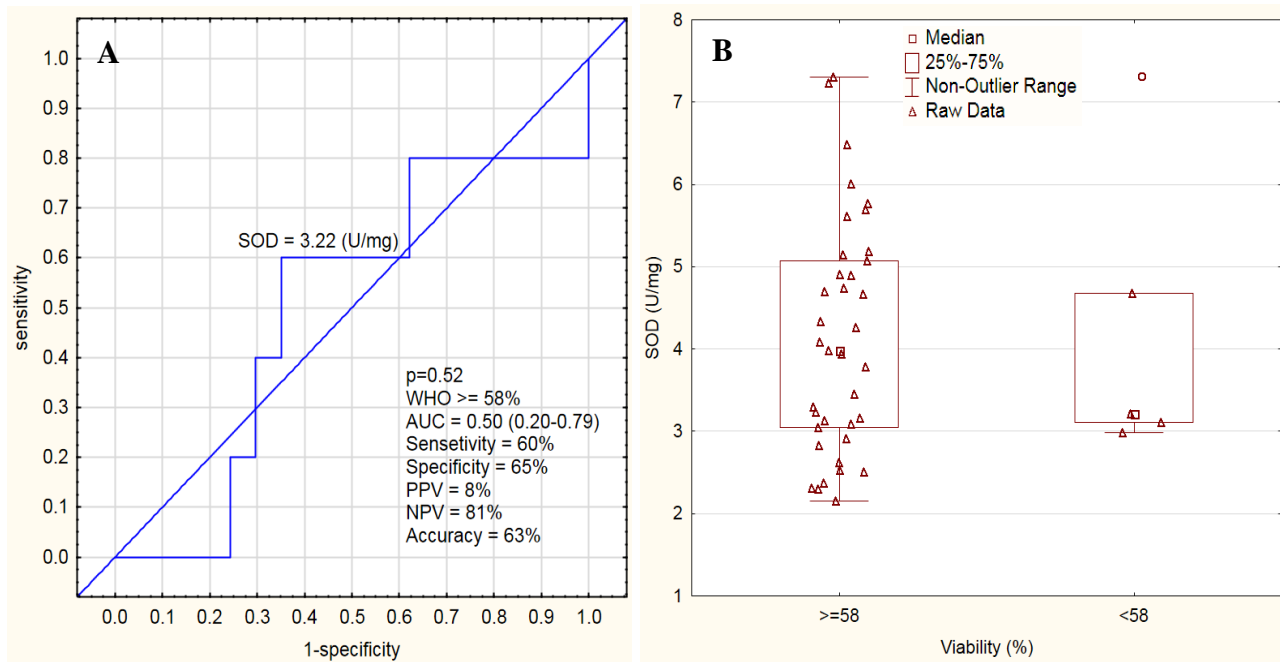


Figure 4. 28: A. Receiver operator characteristic (ROC) curve showing seminal SOD activity (U/mg) cut-off value and the area under the curve. B. Distribution of catalase activity values in samples with normal and below WHO sperm viability reference values. SOD =superoxide dismutase, U = unit, mg = milligram, WHO = World Health Organization, PPV = positive predictive value, NPV = negative predictive value. ≥ 58 , 38 samples, <58; 6 samples.

Chapter 5: Discussion

5.1 Aim I: Effect of abstinence period on semen quality.

Previous studies investigating the effect of ejaculatory abstinence on semen quality have yielded conflicting results due to numerous factors such as large variability of the parameters and small sample sizes. The use of CASA together with advanced measures in the current study is expected to contribute extensively to a better understanding of the potential effect of ejaculatory abstinence on sperm quality using a considerably large population of normozoospermic men. In addition, this study also considered the application of a set of basic semen variables from the first ejaculate in a multiple linear regression model for the prediction of various basic and advanced variables of the second ejaculate.

5.1.1 Ejaculatory abstinence and basic semen parameters

All the results relating to the comparisons between short and long abstinence periods concerning the basic semen parameters will be discussed comprehensively in the following section. This includes interpretation, explanation and the potential mechanisms that are relevant to this study.

5.1.1.1 Semen pH

The slight alkalinity of seminal fluid is essential for neutralizing the acidic environment of the vagina, which is detrimental to spermatozoa (Nakano et al., 2015). Results of the current study showed marginally, but statistically significant increase in semen pH in samples collected after 4 hours compared to those collected after 4 days of abstinence. The mean pH values for semen collected after both short and long abstinence periods remained above the lower WHO reference value of 7.2 (WHO, 2010). Only few studies considered seminal pH as a parameter when investigating the relationship between the abstinence period and semen quality (Choavaratana et al., 2014; Jurema et al., 2005; Valsa et al., 2013). Blackwell and Zaneveld (1992) analysed semen samples from ten men with abstinence periods of 1, 2, 3, 4, 5 and 10 days, and found that seminal pH remained relatively

unchanged. In addition, De Jonge et al. (2004) examined ejaculates from 11 men who had abstained for 1, 3, 5 and 8 days, and reported no significant differences in seminal pH across the four abstinence periods. Similar results were also reported in a recent study by Agarwal et al. (2016) who collected semen samples from seven men each abstaining sequentially for 1, 2, 5, 7, 9 and 11 days, and observed that semen pH remained relatively stable but declined significantly after 11 days of abstinence. The scarcity of studies examining seminal pH indicates that the significance of this semen marker has clearly been underestimated.

The scientific base for the association between the length of the sexual abstinence and semen pH is not entirely clear. However, the accumulation of the metabolic end products such as CO₂ within the cauda epididymis could have a role to play in increasing the hydrogen ion levels in the epididymal fluid which may, in turn, contribute to the acidity of the ejaculate. In addition, the epithelium of the cauda epididymis is structured to decrease the pH, which assist in keeping the metabolic rate of unejaculated spermatozoa minimised (Jones and Murdoch 1996; Valsa et al., 2013).

Furthermore, split ejaculation studies, which examine properties of different ejaculate fractions (Valsa et al., 1994; Valsa et al., 2012), have shown that the initial fraction of the ejaculate is primarily made up of spermatozoa suspended in relatively pure prostatic secretion. The subsequent portion contains a combination of both prostatic and seminal vesicle secretions, while the last portion of the ejaculate is predominantly composed of residual spermatozoa suspended in a relatively pure seminal vesicle fluid (Mortimer, 1994). Accordingly, the relatively higher pH values associated with short abstinence may be a consequence of the unequal contribution of the accessory glands to the ejaculate volume in the short vs. long abstinence periods, since the shortage in the prostatic secretions appears to be more pronounced than that of seminal vesicle secretions. In support of this, considerably lower levels of zinc were found in semen samples collected after short abstinence period compared to those collected after long period, with the difference in fructose levels being insignificant (Elzanaty et al., 2005; Mortimer, 1994). Interestingly, a negative correlation was observed between the Zn concentration in seminal plasma and the seminal pH (Lin et al., 2000). Given that seminal plasma

levels of zinc reflect the secretory function of the prostate gland (Alexandrino et al., 2011; Shilstein et al., 2004); the relatively higher pH values observed after short abstinence period may presumably be attributed to the insufficiency of the prostatic secretion to deposit adequate contributions to the volume of the second ejaculate. Moreover, residual accessory gland secretions may make their way into the second ejaculate, further increasing the pH. The reason could be that small volumes of the third and final portion of the ejaculate, which is almost exclusively made of the alkaline seminal vesicle fluid (Mortimer, 1994) may remain in the ejaculatory duct and consequently contribute to the subsequent ejaculate, especially those collected after extremely short periods of abstinence.

5.1.1.2 Semen Volume

Semen volume has consequently been suggested as an early marker of low semen quality even before identifying any abnormality in concentration, motility and morphology of spermatozoa. Semen volume has also been shown to provide a reliable indication of the secretory functions of the accessory glands, particularly the seminal vesicles (WHO, 2010). The volume of ejaculate in this study was significantly decreased after a short period of abstinence compared with a long period. However, even after such an extremely short abstinence period, the mean ejaculate volume remained above the lower reference value of 1.5 mL recommended by the WHO (WHO, 2010). There is robust and consistent evidence for the significant increase in semen volume with increase in abstinence period in men with normal (Agarwal et al., 2016; Mayorga-Torres et al., 2016; Valsa et al., 2012) as well as abnormal semen profiles (Bahadur et al., 2016a; Levitas et al., 2005; Sánchez-Martín et al., 2013). Interestingly, in healthy normozoospermic men, the greatest overall mean of daily increase in semen volume during the first 4 days of abstinence was calculated to be 11.9 % per day (Carlsen et al., 2004). Furthermore, the volume of ejaculate has generally been estimated to increase with an average of 0.4 mL per day as the interval between ejaculations increases and reaches a peak between the fifth and seventh day of abstinence (Ayad et al., 2018). However, only one study (Magnus et al., 1991) failed to show any significant change in semen volume in both normozoospermic and asthenozoospermic populations,

which is likely to be due to the small number of samples investigated, and the protracted period of the short abstinence.

The reduction observed in the ejaculate volume after a shorter abstinence period could be attributed to a continual semen extraction from the epididymal reserve and the sluggish replacement by testicular, epididymal and genital accessory gland secretions of this reserve. It may also be interpreted as an insufficiency of the accessory glands to deposit adequate contributions to the ejaculate volume, particularly the seminal vesicles and the prostate gland, which are the major contributors to the ejaculate volume. The epithelial tissues of these organs are targeted by androgen, which is thought to regulate their mRNA production as well as the synthesis of rough endoplasmic reticulum, thereby enhancing the production of seminal plasma proteins (Fawell and Higgins, 1984). Improved secretory capacities of seminal vesicles and prostate gland has been associated with higher endogenous serum testosterone levels in rats (Zanato et al., 1994), and men (Gonzales, 1994). In addition, higher testosterone serum levels have been reported following a prolonged abstinence period compared with a shorter abstinence (Jiang et al., 2003). Therefore, the potential stimulating effect of testosterone on the major accessory glands associated with long abstinence periods may contribute to the increased semen volume after prolonged abstinence periods.

In order to predict the semen volume of the second ejaculate (dependent variable) from various parameters obtained from the first ejaculate, subset linear regression analysis was performed to find the best independent variables to achieve this. Three independent variables from the first ejaculate namely volume, progressive motility and VAP were identified through best subset linear regression as indicated in Table A1 (Appendix). The volume of the first ejaculate, as an independent variable, appeared as a predictor in all 20 of the top best models, thus identifying it as a particularly important predictor. Furthermore, it is shown that the semen volume of the second ejaculate could be predicted with relative accuracy, as nearly 63 % proportion of variance ($R^2 = 0.627$) could be accounted for, while the new case predictability had a 56 % accuracy according to the cross validation R^2 of 0.56. However, in terms of the physiological predictability relationship, the robustness of this test must be

validated using bigger sample sizes and wider variation of individuals, including samples from infertile patients.

5.1.1.3 Sperm Concentration

The concentration of spermatozoa in semen, expressed as millions per millilitre, is a critical indicator of semen quality and a prognostic factor for fertility potential (Guzick et al., 2001). However, it is not recommended as an accurate measure of spermatogenesis because it is influenced by the volume of secretions from the accessory sex glands in which the concentrated epididymal spermatozoa are diluted during ejaculation (WHO, 2010). The T.S.C. in the ejaculate, expressed as millions per total ejaculate and obtained by multiplying the sperm concentration by the semen volume, is suggested to be a better marker for the evaluation of spermatogenesis (Amann, 2009).

Results of the present study showed that the T.S.C. as well as sperm concentration were significantly decreased in samples collected after short abstinence compared to those collected after long abstinence. Even with only 4 hours of abstinence, the mean values of these two parameters did not decline to below the lower threshold values of 15×10^6 spermatozoa/mL and 39×10^6 spermatozoa/ejaculate respectively, as recommended by the WHO (WHO, 2010). The period of abstinence has usually been associated with an increased total number (Bahadur et al., 2016a; Marshburn et al., 2014; Sunanda et al., 2014) and higher concentration of spermatozoa (De Jonge et al., 2004; Marshburn et al., 2010; Sukprasert et al., 2013).

During sexual inactivity, an estimated 400 million spermatozoa are reserved within the epididymis with the majority stored in the cauda epididymis and lesser in the caput and corpora with an average of 90 million in each of these sections. The paired vas deferens with its ampulla is estimated to contain about 75 million spermatozoa (Guzick et al., 2001). During the arousal phase, but prior to the emission phase, the population of spermatozoa in the paired ampulla increases dramatically as they move distally towards the urethra (Durairajanayagam et al., 2015). After particularly long periods of abstinence, the bulk of the sperm population in the first ejaculate mainly consists spermatozoa stored

in the ampulla and vas deference, and partly those from the cauda epididymis. Consequent ejaculates in quick successions are typically characterized by a lower T.S.C. as the residual spermatozoa are flushed from the proximal cauda and corpus, and thereafter from the caput (Valsa et al., 2013), all of which contain much lower sperm reserves (Guzick et al., 2001). Despite these findings, Bahadur et al. (2016a) interestingly suggested that “combining the initial and consecutive ejaculates allows for a potential shift of severe oligozoospermia towards the normozoospermia”. This approach may lead to a change in the treatment strategies by possibly avoiding testicular biopsies and IVF procedures, if more sperm can be accumulated to make IUI procedures clinically viable (Bahadur et al., 2016b).

The observed consistent positive association of sperm concentration and total count with increasing abstinence periods can be ascribed to daily sperm production, which is determined to be approximately $130\text{-}270 \times 10^6$ per day (Amann, 2009). The regulation of testicular functions and spermatogenesis necessitates a complex combination of endocrine and paracrine signals. Relatively higher levels of testosterone are essential for the maintenance and proceeding of spermatogenesis. Serum testosterone levels were shown to fluctuate mainly from the second to the fifth day of abstinence, reaching a peak (about 145 % of the baseline) after the seventh day of abstinence and remaining relatively constant even when the abstinence period was further prolonged (Jiang et al., 2003). Simultaneously, according to the systematic review performed by Ayad et al. (2018), the largest increase in the overall mean of T.S.C. observed in 18 studies was reported when the abstinence period extended from 6-7 days to >7 days (Ayad et al., 2018).

Nearly 44% percent ($R^2 = 0.439$) of the proportion of variance in the sperm concentration of the second ejaculate, as a dependent variable, could be predicted from an independent set of first ejaculate parameters, while new cases could be predicted with approximately 37 % accuracy according to the cross validation R^2 of 0.37. Sperm concentration, progressive motility and BCF of the first ejaculate were identified as best independent variables (Table A2, Appendix). The sperm concentration of the first ejaculate as an independent variable occurred in 19 of the top 20 best models, thereby indicating

that sperm concentration in the first sample is the major contributor to predicting the concentration in the second sample.

5.1.1.4 Sperm Viability

Sperm viability is one of the parameters that is routinely assessed in basic semen analysis, and is especially recommended in samples where the percentage of sperm motility is less than 40 % (WHO, 2010). The percentage of viable spermatozoa in this study was not significantly different between the short and long abstinence periods. The mean viability percentage remained above the WHO lower reference value of 58 % (WHO, 2010) even after 4 hours of abstinence. These findings are consistent with those of previous studies which also observed slight or no statistically significant association between sperm viability and abstinence period (Agarwal et al., 2016; Choavaratana et al., 2014; De Jonge et al., 2004; Marshburn et al., 2010; Mayorga-Torres et al., 2016). Across the studies reviewed by Ayad et al. (2018), the overall mean percentage of viable spermatozoa was found to peak and remain relatively unchanged between the second and the fifth day of abstinence, and declined thereafter (Ayad et al., 2018).

This study also showed that the proportion of viable spermatozoa in the second ejaculate as a dependent variable could be predicted from the first ejaculate as nearly 50 % proportion of variance ($R^2 = 0.503$) could be accounted for, while new cases could be predicted with 47 % accuracy according to the cross validation R^2 of 0.47. Sperm viability and LIN of the first ejaculate were identified as best independent variables, with sperm viability being the most important predictor as it occurred in all 15 of the top best models (Table A3, Appendix).

5.1.1.5 Sperm Morphology

To be considered morphologically normal, the whole spermatozoon and its three distinct areas, that is, the head, midpiece and the tail, must fit the stringent criteria in terms of their size and shape (WHO, 2010). It has also been reported that morphologically abnormal spermatozoa, with a special focus on the acrosomal region, have a lower chance to bind to the zona pellucida (Garrett et al., 1997). The

results of this study found no significant difference in the percentage of morphologically normal spermatozoa between short and long abstinence. After 4 hours of abstinence, the mean percentage morphologically normal spermatozoa remained above the WHO lower reference value of 4 % (WHO, 2010). These findings are in line with the majority of previous studies, which also reported no significant association between sperm morphology and the period of abstinence (De Jonge et al., 2004; Mayorga-Torres et al., 2016; Sunanda et al., 2014). In contrast, one study reported significantly higher percentages of spermatozoa with tail defects when the abstinence period was extended from 2-3 days to 6-7 days, while the overall proportion of normal morphology did not differ between the two abstinence groups (Elzanaty et al., 2005). Furthermore, Levitas et al. (2005) reported that among mild to moderate oligozoospermic samples, the highest percentage of normal morphology was reported at ≤ 2 days of abstinence but this association was not observed in a normozoospermic population. Interestingly, Bahadur et al. (2016a) recently reported that an extremely short abstinence period of 30 minutes could significantly improve sperm morphology among oligozoospermic men, all candidates for IUI treatment. By contrast, shortening the abstinence duration in normal individuals from 3-5 days to only 18-30 hours resulted in a considerably lower percentage of morphologically normal spermatozoa (Sukprasert et al., 2013). It may therefore be advantageous for patients with oligozoospermia to abstain for shorter periods before sperm collection, as an option in the process of fertility treatment.

Nevertheless, it must be taken into consideration that manual assessment of sperm morphology is a subjective analysis with inter- and intra-laboratory variation. This variability may be attributed to several factors including the use of different fixation and staining techniques (Maree et al., 2010), differences in interpretation (Kruger and Coetzee, 1999) and technician expertise (Barroso et al., 1999). Another important factor that needs to be reiterated is that the WHO guidelines and reference ranges have changed over the years and may thus lead to differences in interpretation (WHO, 2010).

In the current study the percentage of normal sperm morphology of the second ejaculate, as a dependent variable, could be predicted from a set of first ejaculate parameters which could account

for approximately 48 % of the proportion of variance ($R^2 = 0.483$), while a new case could be predicted with 46 % accuracy according to the cross validation R^2 of 0.46. Three independent variables from the first ejaculate, that is, normal morphology, VCL and T.S.C. were shown to be the best possible predictors of the percentage of normal sperm morphology of the second ejaculate through best subset linear regression, as illustrated in Table A4 (Appendix). The percentage of morphologically normal spermatozoa of the first ejaculate displayed the strongest prediction as it appeared in all 20 of the top best models.

5.1.1.6 Sperm Motility

Motility is a critical variable in the evaluation of sperm parameters and is a significant indication of the functional capacity of spermatozoa (Gunalp et al., 2001). The issue of the influence of ejaculatory abstinence on motility characteristics of spermatozoa has been considered in several previous studies, but the results have often been conflicting. In this study, CASA results showed a significant increase in the percentages of total sperm motility as well as progressive motility after short abstinence compared with long abstinence. These findings are in agreement with numerous other studies where the proportions of total motility (Choavaratana et al., 2014; Levitas et al., 2005; Marshburn et al., 2010; Valsa et al., 2013) and progressive motility (Bahadur et al., 2016a; Elzanaty et al., 2005; Sobreiro et al., 2005; Sunanda et al., 2014) were found to improve after shorter abstinence. The overall mean peaks of both total and progressive motility across the studies reviewed by Ayad et al. (2018) were reported after ≤ 1 day of abstinence. On the other hand, other studies failed to identify any significant difference in the percentages of total motility (Agarwal et al., 2016; Mayorga-Torres et al., 2015; Mayorga-Torres et al., 2016; Sánchez-Martín et al., 2013; Sukprasert et al., 2013), and progressive motility (Mayorga-Torres et al., 2015; Mayorga-Torres et al., 2016). Interestingly, the results of the current study are consistent with those reported by Magnus et al. (1991), who found that progressive motility of a normozoospermic population was increased after shortening the abstinence period. However, when Magnus et al. analysed an asthenozoospermic population, no such association

was found, thereby corroborating the findings of the other relevant studies (Cooper et al., 1993; Jurema et al., 2005; Mayorga-Torres et al., 2015; Mayorga-Torres et al., 2016).

These variations might be a result of differences in the populations studied, the small sample size as well as the potential counting and interpretation errors associated with the subjective visual assessment of sperm motility employed in their studies. CASA, in contrast to subjective manual motility estimation, is certainly a powerful approach for the objective assessment of sperm motion. The most recent WHO guidelines on semen analysis nevertheless indicate that the assessment of sperm motility percentage using CASA may be unreliable due to the potential misidentification of particulate debris as immotile spermatozoa (WHO, 2010). However, the SCA 5.4 used in the current study is an up-to-date CASA system equipped with intelligent filters to accurately identify the spermatozoa and eliminate debris and other cells, allowing for more accurate and objective assessment of sperm motility. Therefore, it is suggested that the current guidelines concerning the employment of CASA in semen analysis should be reconsidered.

The subset linear regression showed that the percentage of sperm progressive motility of the second ejaculate as a dependent variable could be predicted from a set of the first ejaculate parameters, which could account for nearly 58 % proportion of variance ($R^2 = 0.0.576$), and a new case could be predicted with 55 % accuracy according to the cross validation R^2 of 0.55. Both progressive motility and ALH of the first ejaculate were identified as best independent variables (Table A5, Appendix), and sperm progressive motility of the first ejaculate as an independent variable appeared in all 14 of the top best models, showing that it is the principal predictor. Furthermore, two independent variables from the first ejaculate namely percentage of total motility and ALH were identified through best subset linear regression for the prediction of proportion of total motility in the second ejaculate from the first, as shown in Table A6 (Appendix). The proportion of sperm total motility in the first ejaculate as an independent variable emerged as predictors in all 14 of the top best models, thus identifying it as the most significant predictor. The percentage of sperm total motility of the second ejaculate could, thus, be predicted with approximately 55 % accuracy as a substantial proportion of

variance ($R^2 = 0.549$) could be accounted for, and new cases could be predicted with 52 % accuracy according to the cross validation R^2 of 0.52.

Sperm velocity is considered one of the most important determinants of sperm competition outcomes and is vital in predicting the fertilization success (Tourmente et al., 2011). The CASA results of the present study showed a significant increase in the proportion of Type A spermatozoa after short abstinence compared with long abstinence, whereas the proportions of type C and type D spermatozoa were significantly lower after short abstinence. The proportion of type B spermatozoa was not significantly different between the abstinence periods. Similar trends were reported in a recent study by Bahadur et al. (2016a).

This study also found a significant increase in the kinematic parameters of VCL, VSL, VAP, LIN, STR and BCF after short abstinence compared with long abstinence, while no differences were detected in WOB and ALH. The only other study investigating the impact of ejaculatory abstinence on sperm kinematics, among other markers of semen quality, had been conducted by Elzanaty et al. (2005). In this study, semen samples collected from patients with a wide age range undergoing infertility assessment were grouped into three categories based on the abstinence period. Significantly higher VSL and LIN values were observed among the group of men with the shorter abstinence while VAP and VCL values, in contrast to the present study, were not significantly different between the abstinence groups. It is worth mentioning that variation in semen characteristics among individuals may enhance the potential for observation bias (Keel, 2006). However, in the current study the ejaculations of both short and long abstinence were collected in quick succession from the same donor, thereby eliminating confounding variables.

The increase in semen volume and sperm concentration with a long abstinence period was accompanied by substantial deterioration in sperm motility characteristics, especially progressive motility and velocity. Although the exact mechanism as to how ejaculatory abstinence may affect changes in semen quality is still largely undefined, a number of possibilities can be suggested and will subsequently be explained in the following section.

Senescence of unejaculated spermatozoa: With increasing abstinence time, older and senescent spermatozoa are gradually accumulating in the epididymis. Under normal conditions, the precise mechanism by which unejaculated senescent spermatozoa are eliminated from the epididymal duct is not fully understood. However, few reports are available regarding the potential role of the epithelial cells and intraluminal phagocytes in the uptake and the disposal of these defective spermatozoa (Alvarez and Obregon, 1995; Holstein, 1978; Sutovsky et al., 2001). The relative contribution of these senescent spermatozoa to the subsequent ejaculation impairs semen quality, unless they are ejaculated and removed from the male reproductive tract at regular intervals (Mortimer, 1994; Sánchez-Martín et al., 2013). Therefore, semen samples collected after prolonged abstinence are generally characterized by substantially higher sperm concentration, but poor quality (Choavaratana et al., 2014; Levitas et al., 2005; Marshburn et al., 2010). Reduction in the storage period within the epididymis may minimize the exposure of unejaculated spermatozoa to motility inhibitory factors and enzymes released from the degenerating cells within the same microenvironment (Valsa et al., 2013).

Effect of sperm motility quiescence factors: During prolonged abstinence periods, spermatozoa stored within the cauda epididymis become more docile whilst immersed in this environment, as they are constantly exposed to various sperm motility quiescence factors (Iwamoto and Gagnon, 1988; Skandhan, 2004). In addition to the inhibitory effect of the relatively acidic epididymal environment on sperm motility, several other factors present in the male reproductive fluids of mammals, have been shown to have the capacity to enforce quiescence on the caudal spermatozoa (Acott and Carr, 1984). In the rat caudal epididymis, sperm motility is suppressed mechanically due to the effect of a high molecular weight glycoprotein known as Immobilin, which raises the viscoelastic drag of the epididymal fluid surrounding the spermatozoa (Usselman and Cone, 1983).

The presence of motility quiescence factors has also been reported in human seminal fluids and suggested to contribute significantly to the etiology of motility impairment of ejaculated spermatozoa. Iwamoto and Gagnon (1988) purified a seminal plasma motility inhibitor (SPMI) and examined its

potential effect on ejaculated human sperm motility. This study showed a considerable decrease in the motility and velocity of the spermatozoa incubated with the SPMI in a dosage and time-dependent manner. Although the association between abstinence period and these motility quiescence factors has yet to be elucidated, it can be speculated that the release of spermatozoa through more frequent ejaculations could reduce the action of these inhibitory factors, as their concentrations decrease to basal levels. This may eventually help to reduce the severity of these factors and minimize their adverse effects on sperm motility.

Effect of crowding and limited space: Previous studies have shown that the total number of spermatozoa accumulated in the cauda epididymis and subsequently released in the ejaculate may vary substantially even among individuals sharing the same period of abstinence (Carlsen et al., 2004; Levitas et al., 2005; Marshburn et al., 2014; Sunanda et al., 2014; Valsa et al., 2013). In the current study, long abstinence-associated increase in sperm concentration was accompanied by a relative, though not significant, elevation in semen viscosity. With increased viscosity, the sperm cells become closely packed with restricted fluid space available for spermatozoa to move freely. This is presumed to be supported by the findings of previous studies, which have shown a negative correlation between sperm count and motility characteristics in patients with a remarkable increase in sperm concentration (Amelar et al., 1997; Patil et al., 2013).

Depletion of energy source: Fructose, the primary carbohydrate formed in semen, is an essential source of energy for sperm motility (Gonzales, 2001; Gonzales and Villena, 2001). The entire process of fructolysis, in bull sperm for instance, is estimated to produce about 6×10^6 ATP molecules per sec for each motile sperm, with an average of 2 mg fructose being utilized by 1000×10^6 sperm per hour (Rikmenspoel et al., 1969). Interestingly, an inverse association has been reported between seminal plasma levels of fructose and sperm concentration (Ahmed et al., 2010; Rajalakshmi et al., 1989). Thus, it can be assumed that as the number of spermatozoa increases, as in during prolonged abstinence, the amount of fructose available for each individual spermatozoon decreases, leading to the depletion of energy necessary for the sperm to move spontaneously. This may rationalize the

positive correlation between seminal fructose levels and sperm motility, as was previously reported (Patel et al., 1987).

Accumulation of Frequent heat exposures: Reserved spermatozoa are believed to be highly sensitive to increased external temperature (Mortimer, 1994); hence, they are situated in the epididymal cauda, which is the coolest part of the scrotum (Bedford, 1978). The severity of the detrimental effect of increased scrotal temperature on the unejaculated sperm cells has been shown to vary depending on the intensity of the heat stress, as well as the frequency and the period of exposure (Durairajanayagam et al., 2015; Rao et al., 2015). Therefore, extending the abstinence period may possibly enhance the susceptibility of unejaculated spermatozoa to recurrent genital heat exposure.

Repeated exposures to temperatures higher than the optimum scrotal temperature may adversely affect the motility characteristics of epididymal spermatozoa via altering the properties of the motor apparatus of the sperm flagellum. This may presumably occur by increasing the probability of thermal denaturation of proteins involved in the motor apparatus of the sperm flagellum (Saikhun et al., 1998). In addition, substantial changes in the membrane phospholipid architectures of cauda epididymal spermatozoa have also been reported in male mice subjected to few degrees above the core body temperature for three consecutive days (Wechalekar et al., 2010). Frequent and accumulating heat exposures may also cause changes in the composition of the epididymal fluid, which in turn influence its normal function. An abnormal epididymal milieu may reduce the time needed for sperm maturation via accelerating the epididymal transit time, thereby leading to increased proportions of spermatozoa with impaired motility in the ejaculate (Rao et al., 2015). Consequently, reducing the abstinence period may minimize the frequency and time span of heat exposure, thereby leading to improved motility.

5.1.2 Ejaculatory abstinence and advanced semen parameters

Conventional semen parameters provide the essential information on which clinicians base their preliminary diagnosis (Giwerzman et al., 2010). Approximately 25-40 % of idiopathic infertile males

have been reported to have normal semen profiles (Gudeloglu et al., 2015). Therefore, a range of advanced sperm quality parameters have been developed to circumvent the limitations of the conventional semen analysis (Erenpreiss et al., 2006). In the following section, all the results relating to the influence of abstinence period on the advanced semen parameters measured will be discussed comprehensively. This includes interpretation, explanation and the potential mechanisms involved.

5.1.2.1 Acrosome Reaction

The percentage of acrosome-intact spermatozoa was not significantly different between short and long abstinence periods. However, there are no previous reports in the existing literature with which to compare these results. In clinical settings, the rate of spontaneous (premature) acrosome loss may provide little indication on the fertilizing ability of spermatozoa, unless the values are particularly high (Pilikian et al., 1992).

Epididymal spermatozoa are characterized by low membrane fluidity and limited intracellular calcium levels, thus, their acrosome integrity remains almost constant throughout the epididymal duct (Fàbrega et al., 2012). The inability of most mammalian spermatozoa to undergo the acrosome reaction during epididymal passage and storage is generally attributed to the presence of decapacitation factors in the epididymal milieu (Thomas et al., 1984). For instance, the acrosome stabilizing factor (ASF), which was isolated from the epididymal fluid and seminal plasma of some mammals, is thought to be synthesized and released by principle cells throughout the epididymal duct. The ASF has been shown to play a role in stabilizing the spermatozoa and preventing the process of acrosome exocytosis during epididymal transit and storage. The average levels of ASF were found to be considerably varied among different segments of the epididymis with lowest ASF levels being observed in the caput and corpus epididymal fluid, whereas the highest level was in the cauda epididymal fluid and vas deferens. In the cauda epididymal fluid, the ASF consists about a quarter of the total proteins in this segment (Reynolds et al., 1989; Thomas et al., 1984).

As mentioned earlier, spermatozoa stored in the vas deferens and cauda are released during the first ejaculation, whereas the remainders from the cauda and corpus and later from the caput are released in the subsequent ejaculations (Valsa et al., 2012). During prolonged sexual abstinence, spermatozoa stored in the cauda are increasingly susceptible to various acrosome reaction inhibitory factors. However, consequent ejaculates in quick successions may presumingly decrease the effectiveness of these factors as their concentrations are minimised to basal levels. This could be a possible reason for the relatively, but not significantly, lower percentage of acrosome-intact spermatozoa observed in the current study after the short abstinence period.

5.1.2.2 DNA Fragmentation

Assessment of sperm DNA integrity, in addition to routine semen analysis, provides further valuable information about sperm quality as well as pregnancy outcomes (Borini et al., 2006; Tesarik et al., 2004). It has been shown that high proportions of spermatozoa with DNA fragmentation above 20 % increase the risk of infertility regardless of having normal basic semen parameters (Giwercman et al., 2010). The current study showed no significant difference between short and long abstinence periods regarding the percentage of sperm DNA fragmentation. Conflicting results have been reported in this regard. The finding of the current study concurs with a number of previous studies (De Jonge et al., 2004; Gosálvez et al., 2011; Mayorga-Torres et al., 2015). Other studies, however, reported increased levels of DNA damage with increasing abstinence period (Agarwal et al., 2016; Sánchez-Martín et al., 2013; Sukprasert et al., 2013). Surprisingly, an extremely short abstinence period of only two hours was reported to be associated with higher levels of sperm DNA fragmentation (Mayorga-Torres et al., 2016). The latter might be the result of a small sample size ($n=3$) as well as the large variations in values obtained.

The use of different methodologies (e.g. Comet assay, sperm chromatin structure assay and sperm chromatin dispersion test) in the evaluation of sperm DNA damage may also be responsible for such contradictory findings among studies, as different techniques may assess different aspects of chromatin integrity (Ribas- Maynou et al., 2013). The TUNEL assay used in this study allows for the

measurement of both single and double DNA strand breaks, wherein spermatozoa are scored as TUNEL positive or negative according to their fluorescence intensity detected by flow cytometry.

As depicted in Table A7 (Appendix), five independent CASA variables from the first ejaculate, i.e. progressive motility, ALH, BCF, sperm concentration and T.S.C. were shown through best subset linear regression to be useful for the prediction of the percentage of sperm DNA fragmentation of the second ejaculate from the first ejaculate. The kinematics ALH and BCF of the first ejaculate, as independent variables, were found to be the most important predictors as they appeared in all 20 of the top best models. Thereby, the percentage of spermatozoa with DNA fragmentation in the second ejaculate could be predicted with relatively high accuracy as approximately 75 % proportion of variance ($R^2 = 0.739$) could be accounted for, and new cases could be predicted with 42 % accuracy according to the cross validation R^2 of 0.42.

5.1.2.3 ROS generation and oxidative stress markers

$O_2^{\cdot-}$ is the major initial form of ROS produced by sperm. The physiological or pathological effects of ROS on sperm function are thought to be influenced by the type and the amount of reactive species involved (Kothari et al., 2010), and importantly the period of exposure in the context of abstinence. In the current study intracellular $O_2^{\cdot-}$ levels, although not significant, were slightly lower after short abstinence compared to longer abstinence. Decreased time spent in storage within the epididymis reduces the production of by-products of aerobic metabolism, and minimises the susceptibility of spermatozoa to external factors, such as heat exposure, leading to less ROS production.

Three studies (Agarwal et al., 2016; Mayorga-Torres et al., 2015; Mayorga-Torres et al., 2016) are available on the relationship between the abstinence period and sperm intracellular ROS production, while only one study examined the relationship in terms of seminal ROS concentration (Desai et al., 2010). These studies consistently reported no association of abstinence period with either intracellular ROS production or seminal ROS levels. However, among the relevant studies a general trend of reduction, albeit non-significant, was observed in intracellular ROS levels after short abstinence in

comparison with long abstinence, as shown in the current study. Interestingly, when four repeated ejaculates were collected on the same day at two-hour intervals, a significant reduction in intracellular ROS production was observed in the fourth ejaculate compared with the initial one obtained after 3 to 4 days of abstinence (Mayorga-Torres et al., 2016).

The subset linear regression analysis, in the present study, showed that none of the independent basic semen variables from the first ejaculate could predict sperm intracellular $O_2^{\cdot-}$ levels (dependent variable) in the second ejaculate as shown in Table A8 (Appendix).

During their maturation and storage, spermatozoa are continuously susceptible to oxidative damage induced by intracellular and extracellular reactive species. Spermatozoa are highly sensitive to ROS damage by lipid peroxidation due to their membranes being highly rich in PUFA (Du Plessis et al., 2015). In addition, spermatozoa have limited intracellular enzymatic defense against oxidative stress, partly due to cytoplasmic extrusion during spermatogenesis. This deficient capacity is effectively compensated for by a group of cellular detoxifying enzymes with powerful antioxidant properties including SOD and CAT found within the seminal plasma (Du Plessis et al., 2010). Activities of these enzymes in seminal plasma have been related to spermatozoa's functional capacity (Foresta et al., 2002; Idriss et al., 2008). The current study found that seminal plasma TBARS levels as well as CAT activity were not significantly different between the short and long abstinence periods, whereas the SOD activity was significantly higher after short period of abstinence.

Surprisingly, only one study had examined the influence of ejaculatory abstinence period on seminal plasma antioxidants and lipid peroxidation of the sperm membrane (Marshburn et al., 2014). By analysing ejaculates of forty men undergoing IUI, Marshburn et al. observed a significant improvement in the total antioxidant capacity of seminal plasma after one day of abstinence compared to four days whereas, as shown in the present study, no significant difference was found with regards to the lipid peroxidation of the sperm membrane between the two abstinence periods. They therefore suggested that short abstinence-related increase of total antioxidant capacity in seminal plasma could

defend spermatozoa against oxidative stress through a mechanism that is independent of lipid peroxidation.

The current study has expanded these findings by investigating the seminal SOD and CAT activities in addition to the sperm intracellular $O_2^{\cdot-}$ levels and the amount of membrane lipid peroxidation as assessed by TBARS. In the present study, a long abstinence-associated increase (insignificant) in sperm intracellular levels $O_2^{\cdot-}$ was accompanied by a depletion of seminal SOD and CAT activity, apparently through their reductions of $O_2^{\cdot-}$ and H_2O_2 respectively. It can therefore be assumed that the capacities of SOD and CAT to reduce oxidative stress were being overwhelmed as lipid peroxidation was still occurring as indicated by the relatively increased levels of seminal plasma MDA (TBARS).

High seminal plasma levels of lipid peroxidation have been reported in infertile men (Colagar et al., 2013; Mahanta et al., 2012). Furthermore, sperm parameters (e.g. progressive motility and morphology) have been associated positively with SOD activity and negatively with lipid peroxidation (Atig et al., 2012; Fazeli and Salimi, 2016; Marzec-Wróblewska et al., 2011; Murawski et al., 2007). A negative association was also reported between SOD activity and MDA levels in seminal plasma, suggesting the importance of this enzyme in protecting against lipid peroxidation (Tavilani et al., 2008). Accordingly, reducing the residence time of spermatozoa within the epididymis, by shortening the abstinence period in this study, would promote the antioxidant defences in the semen and protect spermatozoa from the membrane peroxidative damage.

Three independent basic semen variables from the first ejaculate namely semen volume, pH and sperm concentration were identified through best subset linear regression for the prediction of TBARS levels of the second ejaculate (Table A9, Appendix). The semen pH of the first ejaculate as an independent variable occurred as a predictor in all 19 of the top best models, identifying it as a very strong predictor. Seminal plasma TBARS levels of the second ejaculate could be predicted with relative accuracy since nearly 62 % of proportion of variance ($R^2 = 0.614$) could be accounted for, while a new case could be predicted with only 33 % accuracy according to the cross validation R^2 of

0.33. For CAT activity, the subset linear regression displayed that seminal plasma CAT activity of the second ejaculate, as a dependent variable, could be predicted from the first ejaculate with approximately 67 % accuracy as a large proportion of variance ($R^2 = 0.67$) could be accounted for, and a new case could be predicted with 52 % accuracy according to the cross validation R^2 of 0.52. The T.S.C. and normal morphology of the first ejaculate were found to be the best independent variables (Table A10, Appendix), with sperm normal morphology of the first ejaculate occurring in all 20 of the top best models. Furthermore, two independent basic variables from the first ejaculate namely semen pH and sperm viability were identified through best subset linear regression for the prediction of seminal plasma SOD activity in the second ejaculate (dependent variable) from the first ejaculate, as shown in Table A11 (Appendix). Both of these independent variables showed to be the best predictors as they occurred in all 18 of the top best models. Seminal plasma SOD activity of the second ejaculate could, thereby, be predicted with relatively poor accuracy as only 19 % proportion of variance ($R^2 = 0.185$) could be accounted for, while new cases could be predicted with only 7 % accuracy according to the cross validation R^2 of 0.07.

5.2 Aim II: Relationship between basic and advanced semen parameters

This study has effectively used multivariate analysis in establishing a statistical correlation between various semen analysis parameters and a set of modern markers of semen quality. Despite the great potentials of advanced semen parameters, this study also considered the possibility of determining cut-off values for advanced semen parameters from the basic measurements, which are affordable, undemanding, and having established reference values.

5.2.1 Intracellular O_2^- levels

In conditions where the intracellular redox homeostasis is disturbed, ROS become highly reactive and instigate peroxidative damage, which adversely affect sperm quality (Du Plessis et al., 2015; Sharma et al., 2012). In the current study, a significant and negative correlation was observed between the proportion of morphologically normal spermatozoa and intracellular O_2^- levels. These findings

are consistent with previous studies also showing an inverse association between sperm morphology and ROS production (Aziz et al., 2002). Likewise, substantially higher levels of ROS were reported in teratozoospermic samples compared with normozoospermic controls (Agarwal et al., 2014a). The link between abnormal sperm morphology and ROS overproduction is generally attributed to the presence of excess residual cytoplasm in the midpiece due to deficient cytoplasmic extrusion following spermiation (Aitken, 1997). NADPH oxidase 5 (NOX5), a novel NADPH oxidase responsible for the generation of $O_2^{\cdot-}$ in a calcium-dependent manner, has recently been reported to have a significant positive correlation with the incidence of sperm abnormal morphology (Agarwal et al., 2014b), which further support the negative correlation observed in the current study between sperm normal morphology and $O_2^{\cdot-}$ levels. In addition, abnormal spermatozoa are believed to contribute considerably to ROS production (Gil-Guzman et al., 2001; Oborna et al., 2009). By implication, the relationship between increased ROS levels and sperm morphological abnormality appears to be a vicious cycle indicating a cause and effect relationship that warrants further studies.

Intracellular $O_2^{\cdot-}$ levels in this study were negatively, although not significantly, correlated with the proportions of total and progressive motility as well as rapid spermatozoa. The non-significant correlation between intracellular sperm ROS and motility has also been revealed by Pasqualotto et al. (2000). However, several studies have shown that increased levels of seminal ROS were significantly correlated with decreased motility parameters, including total motility, progressive motility and rapid motility (Agarwal et al., 1994; Aziz et al., 2004; Khosravi et al., 2014; Padron et al., 1997). It is important to note that these studies have mainly focused on extracellular ROS in seminal plasma, while the sperm intracellular ROS has apparently been disregarded. Interestingly, exogenous ROS has been suggested to cause more serious adverse effects on sperm quality compared to equivalent levels of endogenous sperm ROS (Shi et al., 2012). In the current study, flow cytometry was used to determine sperm intracellular levels of $O_2^{\cdot-}$. Flow cytometry assay has been recognized as highly reproducible technology for the measurement of specific intracellular ROS in spermatozoa with a greater degree of accuracy (Mahfouz et al., 2009; Ghaleno et al., 2014).

Despite not observing a significant correlation between $O_2^{\cdot-}$ levels and sperm motility, significantly higher $O_2^{\cdot-}$ levels were found in samples with progressive and total motility below that of the WHO reference values compared to those with normal values ($p < 0.05$). As depicted in Figure 4.20 A, the optimal cut-off value to distinguish between asthenozoospermic and men with normal motility was 227 MFI using flow cytometry techniques. At this cut-off value, the positive and negative predictive values of the test were 44 % and 97 % respectively. In addition, the WHO minimum reference value of 32 % for progressive motility was associated with a 128 MFI as a cut-off value for intracellular $O_2^{\cdot-}$, with a sensitivity and specificity of 100 % and 65 % respectively and 0.79 as AUC (Figure 4.21 A). The distribution of intracellular $O_2^{\cdot-}$ levels for the two groups above and below the WHO reference values for total motility and progressive motility are given in Figures 4.20 B, and 21 B respectively.

This study also revealed a significant negative correlation between sperm intracellular $O_2^{\cdot-}$ and the kinematic parameters VCL, VAP and ALH. These observations suggest that increased concentrations of intracellular $O_2^{\cdot-}$ could initiate alterations in sperm swimming patterns, after which sperm quality may further deteriorate. The VAP is considered an important indicator of the forward swimming speed of spermatozoa; it estimates the time-averaged velocity of the sperm head along its average trajectory (Nagy et al., 2015; Pereault, 2002). The observed inverse relationship between sperm $O_2^{\cdot-}$ levels and VAP may demonstrate the possible role of this anion in the decline of the actual rate of sperm forward movement within the female reproductive tract.

On the other hand, the parameters VCL and ALH describe the movement characteristics of the sperm head, which depend on the pattern of the flagellar beating (Mortimer and Mortimer, 1990; Rathi et al., 2001). Considerable increases in the values of VCL and ALH are generally identified to be characteristic signs of sperm hyperactivation at the site of fertilization (Mortimer and Mortimer, 1990). At low concentrations, the role of $O_2^{\cdot-}$ in the initiation of hyperactivation has been recognized (Lamirande and Gagnon, 1993). However, the strong negative correlation between $O_2^{\cdot-}$ and these kinematics suggests that excessive production of sperm intracellular $O_2^{\cdot-}$ may contribute to decreased

hyperactivated motility, which is critical to the success of fertilization. Further studies are needed to elucidate whether this is an actual cause and effect relationship or just a statistical correlation.

Intracellular O_2^- is not the only oxygen species affecting sperm motility, the exposure to exogenous H_2O_2 has also been shown to have an inhibitory effect on various sperm motility and kinematic parameters in a dose and time dependent manner (Maia et al., 2014). Furthermore, a recent study conducted by Takeshima et al. (2017) showed a significant and inverse correlation between the overall ROS levels and the parameters VCL, VSL and ALH as measured by CellSoft 3000™. Conversely, when sperm motion parameters were assessed using the sperm motility analysing system, a new CASA system, only ALH showed a strong correlation with ROS levels, while VCL and VSL were not correlated with ROS levels (Takeshima et al., 2017).

In contrast to the above cited findings, the current study observed no correlation between intracellular O_2^- levels and STR, while the correlation with regards to the VAP was highly significant. It is, however, worth mentioning that these parameters might not be comparable among different CASA systems due to variations in the video sampling rate, the quality of the camera and the algorithms used by CASA systems (Lu et al., 2014; Mortimer, 2000; Mortimer and Swan, 1999). This necessitates appropriate standardization of CASA systems to improve the consistency and reliability of sperm motion measurements across laboratories. For the current study, the video sampling rate of 50Hz was used as recommended by ESHRE guidelines for the utilization of CASA (Mortimer et al., 1998).

5.2.2 Seminal plasma lipid peroxidation

A statistically significant negative correlation was found between TBARS levels and seminal plasma pH. The preservation of the semen pH within its reference ranges (7.2–8.2) is of great importance for the regulation of various physiological sperm functions (Zhou et al., 2015). This correlation could possibly be explained by the fact that at physiological levels of pH in seminal plasma MDA, the major end-product of lipid peroxidation, is present as an enolate ion with low reactivity. However, lowering

the pH causes the formation of highly reactive compound known as beta-hydroxyacrolein, which can react with other molecules in the vicinity and cause a considerable increase in lipid peroxidation (Papac-Milicevic et al., 2016). Furthermore, the accumulation of MDA in a highly acidic milieu results in the formation of long oligomers, which causes hydrolytic cleavage of recently produced MDA oligomers. This process ends with the formation of additional highly immunogenic epitops on major cellular macromolecules that play a part in secondary deleterious reactions (Ayala et al., 2014; Papac-Milicevic et al., 2016).

Seminal plasma TBARS levels were significantly and negatively correlated with the proportion of rapid spermatozoa. A similar, though non-significant, trend was observed with regards to the proportions of progressive and total motility, whereas the correlation of TBARS levels with the proportions of medium and slow spermatozoa were significant and positive. The negative correlation between lipid peroxidation and sperm quality parameters of motility has been reported in a number of studies (Akbari et al., 2010; Colagar et al., 2013; Patel et al., 2009). Interestingly, substantially elevated concentrations of MDA were found in sperm pellet suspensions (Suleiman et al., 1996; Tavilani et al., 2005) and seminal plasma (Colagar et al., 2013) of patients with asthenospermic compared to normozoospermic fertile men. In addition, the *in vitro* exposure of human spermatozoa to electrophilic lipid aldehydes, such as acrolein and 4-hydroxynonenal (4HNE) produced by lipid peroxidation, resulted in a significant decline in both total and progressive motility percentage, while sperm viability was not compromised (Aitken et al., 2012). The findings of the current study also show that the detrimental effect of lipid peroxidation on sperm motility could occur even before cell death was observed.

This study also found a statistically significant negative correlation between TBARS levels and the sperm kinematics VCL, VSL, VAP and BCF. Similar but non-significant trends were observed with regards to percentage of ALH. The parameters VCL, VSL and VAP are measures of sperm progressive velocity and are revealed to play a vital role in sperm competition (Malo et al., 2005). They have also been suggested as potential reliable indicators of male fertility (Farooq et al., 2017;

Nagy et al., 2015; Santolaria et al., 2015). BCF is one of the useful parameters that contribute substantially to the overall sperm linear progression. It indicates the rate at which the curvilinear path crosses the average path; however, it may vary in value depending on the VAP setting on the CASA instrument (King et al., 2000; Lu et al., 2014). The sensitivity of these parameters to the deleterious effects of lipid peroxidation appears to be higher than that of the percentage motility, which was not correlated with TBARS levels in this study.

Although the concentration of seminal plasma TBARS was not correlated with sperm viability, significantly elevated levels were observed in samples with sperm viability percentages below that of the WHO reference values compared to those with normal values ($p < 0.05$). The optimal cut-off value to distinguish between samples with compromised and normal viability on the basis of TBARS was $9.855 \mu\text{mol/L}$. At this cut-off value, the positive and negative predictive values of the test were 97 % and 36 % respectively. The distribution of TBARS levels for the two groups above and below the WHO viability reference value as well as sensitivity and specificity of the test are given in Figure 4.26 A and B.

In general, the most remarkable effect of lipid peroxidation on cellular function is related to the physicochemical properties of cellular and organelle membranes. The specific lipid composition of these membranes is essential to maintain the overall normal sperm function (Agarwal et al., 2014b; Ayala et al., 2014). Spermatozoa membranes contain an extraordinary high content of PUFA (Henkel, 2011). The rate of fatty acid oxidation can differ according to their chain length, degree of unsaturation, and position and configuration of double bonds (DeLany et al., 2000). The distribution of the lipid composition of the sperm plasma membrane has been shown to be regionally different, allowing for the distinctive functions of these domains (Connor et al., 1998). As compared to the sperm head membrane, the sperm tail is estimated to contain substantially higher amounts of total and individual unsaturated fatty acids, mainly docosahexaenoic and arachidonic acids (Connor et al., 1998). The enrichment of the tail membrane with these unsaturated fatty acids contributes to its fluidity and flexibility, which is critical for tail motility (Agarwal et al., 2014b; Rooke et al., 2001).

These fatty acids are also known to be amongst the most biologically active (Else and Kraffe, 2015), which further enhances the susceptibility of the sperm tail to oxidative alterations.

The role of mitochondria in energy production for human sperm motility has been established (Piomboni et al., 2012). Dysfunctions of mitochondrial membrane integrity may represent a major feature of sperm motility impairment (Paoli et al., 2011; Pelliccione et al., 2011). The inner mitochondrial membrane is especially important, as it is the site of electron transport chain and ATP synthesis. This membrane is highly susceptible to lipid peroxidation due to its high PUFA content and close proximity to ROS production within the mitochondria (Castilho et al., 1999; Kalogeris et al., 2014). Unlike the plasma membrane, the inner mitochondrial membrane exclusively contains high levels of the phosphatidylglycerol cardiolipin which is particularly prone to peroxidation (Paradies et al., 2009; Schenkel and Bakovic, 2014). Under normal physiological conditions, the inner mitochondrial membrane is selectively permeable to only neutral small molecules. However, in case of oxidative stress, peroxidation of mitochondrial membrane phospholipids can trigger alterations mitochondrial membrane potential (Piomboni et al., 2012; Wei et al., 2001). These changes have recently been suggested to be an early indicator of motility impairment as they occur in parallel to or even prior to any detectable changes in overall sperm motility (Agnihotri et al., 2016; La Piana et al., 1998). This further supports the importance of rapid progressive motility linear velocity variables as early and sensitive indicators of lipid peroxidation that could be impaired prior to any detectable deterioration in other sperm motion characteristics.

5.2.3 Seminal plasma antioxidant activity

In order to prevent possible cellular damage, excess ROS are constantly scavenged to maintain low concentrations essential for vital cell functions. Seminal plasma is well provided with numerous enzymatic antioxidants including CAT and SOD (Baumber et al., 2000; Fujii et al., 2003; Hsieh et al., 2002). The activity of SOD causes the dismutation of $O_2^{\cdot-}$ radicals and their conversion into O_2 and H_2O_2 . The H_2O_2 produced is subsequently detoxified by CAT to produce H_2O and O_2 (Zelen et al., 2010; Zini et al., 1993).

In this study, seminal plasma CAT activity was correlated significantly and positively with the proportions of total, progressive and rapid motility as well as normal morphology spermatozoa, and significantly and negatively with the proportion of immotile spermatozoa. Similar, but non-significant trends were observed for the SOD activity. These results are in agreement with those reported by Khosrowbeygi et al. (2004), who demonstrated a significant positive correlation between CAT activity and the percentages of progressive motility and normal morphology, while the correlations with SOD activity were not significant. Previous studies have further shown a substantially higher seminal plasma activity of CAT in normozoospermic men as compared to men with asthenozoospermia (Atig et al., 2012; Bykova et al., 2007; Siciliano et al., 2001; Tavailani et al., 2008) or asthenoteratozoospermia (Khosrowbeygi et al., 2004). The observed positive correlations between CAT activity and sperm motility and morphology indicate the importance of this enzyme in the alleviation of ROS-induced oxidative damage, thus reducing the cytotoxicity to spermatozoa.

Available literature provides inconsistent results about the relationship between SOD activity and sperm quality. Some studies have revealed that increased SOD activity in seminal plasma is correlated with a significant improvement in the sperm overall motility (Atig et al., 2012; Murawski et al., 2007; Yan et al., 2014). Other studies have also reported similar, but non-significant results (Hsieh et al., 2002; Khosrowbeygi et al., 2004; Macanovic et al., 2015). The current study did not find a correlation between SOD activity in seminal plasma and sperm motility parameters, while the correlations with VCL and ALH were significantly positive. This suggests that elevated SOD activity in seminal plasma might be an indication of the development of spontaneous premature hyperactivated motility of spermatozoa in the ejaculate. However, sperm regulation is a highly complex process involving multiple variables, thus, the specific role of SOD in the control of sperm motility remains poorly understood and necessitates further research.

The results of the current study exemplify the importance of CAT activity in seminal plasma as a major marker of the antioxidant status of the ejaculate. The ability of CAT to improve sperm motility indicates that H₂O₂ is the ROS that represents the dominant cytotoxic effect upon spermatozoa. In

contrast, the lack of significant correlations between $O_2^{\cdot-}$ levels and sperm motility as well as between SOD activity and sperm motility suggests that $O_2^{\cdot-}$ plays a considerably less cytotoxic role in spermatozoa. These observations are consistent with those obtained by Aitken et al. (1993), Armstrong et al. (1999) and Baumber et al. (2000) who concluded that H_2O_2 is especially responsible for the motility impairment of spermatozoa. These experiments also demonstrated that addition of CAT, but not SOD, to the incubation medium could ameliorate the decline in sperm motility induced by oxidative stress.

There has been a wide consistency among studies about the deleterious effects of H_2O_2 on the quality of sperm movement, with progressive motility being the most affected parameter (Calamera et al., 2001; Du Plessis et al., 2010; Maia et al., 2014). Moreover, the observed decline in sperm motility associated with increased H_2O_2 concentrations was reported to occur in the absence of any measurable signs of plasma membrane damage (Baumber et al., 2000; Calamera et al., 2001). This may substantiate the lack of significant correlation between CAT activity in seminal plasma and sperm viability observed in the current study.

Due to its high permeability across biological membranes, H_2O_2 is assumed to have direct effects on the intracellular enzyme systems (Maia et al., 2014). Several possibilities have been suggested to explain the mechanism through which H_2O_2 deteriorates sperm motility. However, the exact mechanism appears to be not entirely elucidated and merits further investigations. Some studies have ascribed this effect to the potential capacity of the H_2O_2 to deplete intracellular ATP levels (Lamirande and Gagnon, 1993; Armstrong et al., 1999; Bilodeau et al., 2002), leading to impaired phosphorylation of axonemal proteins essential for sperm motility. In contrast, Calamera et al. (2001) demonstrated that the loss of sperm motility after incubation with H_2O_2 was associated with a corresponding increase in intracellular ATP levels due to decreased ATP utilization by non-progressive and immotile spermatozoa. Moreover, excess intracellular concentrations of H_2O_2 have also been assumed to deteriorate sperm motility throughout the inhibition of intracellular ROS scavenging activity (Krzyzosiak et al., 2000; Maia et al., 2014).

5.2.4 Acrosome Reaction

In order to attain full fertilization potential, spermatozoa undergo an exocytotic process characterized by the release of the acrosome's lytic enzymes to enable the fusion of the spermatozoa outer plasma membrane with the oocyte (Esteves and Verza, 2011). The physiological acrosome reaction is an irreversible event that must occur at an appropriate time during the early stages of sperm-egg interaction. Thereby, premature spontaneous acrosome reaction renders the spermatozoon unable to bind and penetrate an oocyte normally (Liu and Baker, 1994; Tesarik, 1989).

The proportion of acrosome-intact spermatozoa, in the current study, was significantly and positively correlated with the proportions of type C, medium and slow spermatozoa, while its correlations with other semen analysis parameters were statistically not significant. These findings appear to contradict those reported by Parinaud (1996), who found that the proportion of spontaneously acrosome-reacted spermatozoa was negatively correlated with progressive motility. Such variation could possibly be due to the utilization of different techniques for the assessment of acrosome integrity, which allows for the detection of different stages of the acrosome reaction (Köhn et al., 1997). In the above cited study (Parinaud, 1996), the acrosome reaction was evaluated using FTTC-GB24 which primarily binds to the inner acrosomal membrane. This assay detects only spermatozoa that have undergone a complete acrosome reaction (Parinaud et al., 1993), since labelling with GB24 lectin necessitates the exposure of this membrane subsequent to a complete loss of the acrosomal content (Fenichel et al., 1989). However, in the present study, identification of acrosome-reacted spermatozoa was performed using FITC-PSA. The PSA lectin is known to bind with the acrosomal matrix (Risopatron et al., 2001) and thereby allows for the identification of both partially and completely acrosome-reacted spermatozoa (Jaiswal et al., 1999; Ozaki et al., 2002).

The observed positive correlation between sperm acrosome integrity and non-progressive motility may presumingly be attributed to an increased fluid resistance to motion and inertia caused by the sperm with larger acrosomal area. In consonance, previous experiment performed in our laboratory showed a negative correlation between the acrosome size and progressive motility in post-swim-up

samples (Murray, 2007). Although prematurely acrosome-reacted spermatozoa might have the ability for forward motility, they are not able to penetrate the zona pellucida and thereby unable to fertilize the egg.

5.2.5 DNA Fragmentation

Despite the remarkable developments in automated semen analyses, the parameters of the conventional semen analysis remain of relatively limited value in clinical practice (Evgeni et al., 2014; Oleszczuk et al., 2013; Vogiatzi et al., 2013). More valuable information about the quality of sperm as well as pregnancy outcome can be obtained via combining results from the conventional semen analysis and validated sperm DNA fragmentation assays (Borini et al., 2006; Evgeni et al., 2014; Fernández-Gonzalez et al., 2008; Sheikh et al., 2008). In this study, a significant negative correlation was observed between the proportion of DNA fragmentation and sperm viability. This result is comparable with the previous findings from Brahem et al. (2012) that revealed a strong negative correlation exists between sperm DNA fragmentation and the percentage of viable spermatozoa. Higher levels of necrozoospermia were also observed among men with elevated levels of sperm DNA fragmentation. Furthermore, the increase in sperm DNA fragmentation induced by long-term *in vitro* incubation was reported to be accompanied by a substantial loss of sperm viability (Muratori et al., 2003). Similarly, a more recent study also demonstrated a strong negative correlation between sperm DNA fragmentation and viability in semen samples with DNA fragmentation rates $\geq 30\%$ (Samplaski et al., 2015). The current study confirms the observations of the above mentioned studies and suggests that sperm viability might represent a potential indicator and a cost-saving measure for semen quality.

Both DNA integrity and viability of spermatozoa are known to be important markers of semen quality. The mechanism responsible for the incidence of DNA fragmentation in ejaculated human spermatozoa is not fully elucidated. One hypothesis proposes DNA breaks within ejaculated spermatozoa to be the result of apoptotic DNA cleavage during the early stages of spermatogenesis (Sakkas et al., 1999). However, at the stage of DNA break down, the apoptotic process is irreversible

and the cells would be eliminated by Sertoli cells prior to ejaculation (Agarwal et al., 2012). Another postulation points to the excessive exposure to ROS as being the causative agent for DNA fragmentation in ejaculated spermatozoa (De Lamirande and Gagnon, 1999). Sperm DNA fragmentation has previously been shown to correlate significantly and positively with the levels of ROS generated by spermatozoa (Barroso et al., 2000). Despite not being able to measure ROS and DNA fragmentation in the same samples, the current study showed a significant negative correlation ($r = -0.33$; $P = 0.04$) between sperm intracellular $O_2^{\cdot -}$ levels and the proportion of viable spermatozoa, thereby, indirectly implying a relationship between ROS and DNA fragmentation.

The current study also observed a positive correlation between the sperm DNA fragmentation and the kinematic parameters, VCL, LIN and STR. This shows that DNA fragmented spermatozoa might still have the capacity for rapid forward motility. However, these spermatozoa might not be able to develop a state of hyperactivated motility at the site of fertilization as was indicated by the negative correlation observed in this study between the proportion of DNA fragmentation and ALH.

Several studies have been undertaken to investigate the possible correlation between sperm DNA fragmentation and a number of semen characteristics such as sperm concentration, motility and morphology. Not all studies, however, have come to the same conclusions. Some studies have revealed poor correlations, as was observed in the present study, between the amount of sperm DNA fragmentation and the basic semen parameters of sperm concentration, motility and morphology (Cassuto et al., 2012; Chenlo et al., 2014; Giwercman et al., 2003; Karydis et al., 2005; Xia et al., 2005). In contrast, other studies have shown significant negative correlations between sperm DNA fragmentation and many of these semen variables (Lin et al., 2008; Sheikh et al., 2008; Zini et al., 2001). More recently, Boushaba and Belaaloui (2015) reported negative correlations between sperm DNA fragmentation and sperm concentration as well as motility, while no significant correlation was found with regards to sperm morphology. As stated in a review by Evgeni et al. (2014), the inconsistencies among different studies concerning the correlation between sperm DNA fragmentation and semen characteristics could be ascribed to several factors. These factors include

various assays used to quantify DNA fragmentation, the use of different techniques for the assessment of semen quality as well as dissimilarities in the characteristics of the populations across studies. The use of flow cytometry-based TUNEL assay in the current study allows for the simultaneous measurement of real DNA damage in a large population of spermatozoa, providing more objective and statistically reliable outcomes (Chenlo et al., 2014; Sharma et al., 2013). Nevertheless, none of the sperm quality parameters (concentration, motility and morphology) displayed a significant correlation with the proportion of DNA fragmented spermatozoa. The result of this study highlights the importance of the TUNEL assay in predicting sperm function, independent of any quantifiable changes in sperm concentration, motility or morphology as measured by CASA in a population of normozoospermic men.

Chapter 6: Conclusion

6.1 Aim I: Effect of abstinence period on semen quality

The data from this study, the most comprehensive study of its kind, challenges the generally accepted guidelines relating to the prolonged ejaculatory abstinence periods of 2-7 days (WHO, 2010), since the results show that 4 hours of abstinence has a beneficial effect on sperm function, as indicated by the significant improvement in sperm total motility, progressive motility and velocity parameters. It is also worth mentioning that even after an extremely short abstinence period of only 4 hours, the mean values of all the basic semen parameters always remained above the lower reference limits as recommended by the WHO in its latest version. In addition, the use of best subset linear regression models enabled, for the first time ever, the predicting of various basic and advanced semen parameters of the second ejaculate (4 hours of abstinence after the first ejaculate) from a set of basic semen parameters obtained from the first ejaculate, which was collected after 4 days of abstinence. Interestingly, each basic dependent variable of the second ejaculate was shown to be particularly predictable by the same basic independent variable measured in the first ejaculate.

Future studies involving biomarkers of epididymal, prostate and seminal function might shed further light on these findings. Despite the fact that this study was performed on normozoospermic men, future studies using infertile men might yield similar findings that could lead to the employing of short abstinence as a strategy to improve the outcome of Assisted Reproductive Technology (ART) and fertility preservation. Therefore, shortening the abstinence period prior to semen collection may be a potential strategy to improve sperm quality. It is thus recommended that the current guidelines regarding the prescribed abstinence period be revisited

6.2 Aim II: Relationship between basic and advanced semen parameters.

The combination of both basic and advanced markers of semen quality remains important to improve the diagnostic and prognostic values of semen analysis. The correlations observed in this study indicate that changes in the measurements of basic semen analysis are related to alterations in the advanced functional semen parameters. Sperm morphology, viability motility and velocity variables were shown to have significant correlations with oxidative stress markers, while sperm DNA fragmentation was correlated with sperm viability and various kinematic characteristics. The optimal individual semen marker that provides the most accurate prediction of the fertilization potential of human spermatozoa remains a controversial issue. However, the use of ROC curve analysis in this study enabled defining the cut-off values for intracellular $O_2^{\cdot-}$ levels (227 MFI) which could distinguish between asthenozoospermic and normozoospermic samples. Furthermore, the cut-off value of 9.855 $\mu\text{mol/L}$ for seminal plasma TBARS levels of was shown to differentiate between samples with compromised and normal viability. This information is of great importance as the cut-off values are highly valuable when identifying the difference between normozoospermic individuals and patients with different abnormalities. While further and larger studies are needed, the correlations between basic and advanced semen parameters observed in this study could possibly assist in limiting the necessity for advanced sperm functional assays, which are complex, highly expensive and lack universal standardization. The prediction of advanced variables from the core basic parameters would also enhance the applicability of basic semen analysis, which remains the bedrock of any semen diagnosis, as a more cost-effective and efficient approach for the diagnosis of idiopathic and unexplained male infertility.

6.3 Limitations/recommendations of the study

Although the study has successfully reached its aims and objectives, there is always opportunity for improvement and a number of shortcomings and limitations were retrospectively identified. First, the study focused on; therefore, all the donors included were healthy, normozoospermic and potentially fertile males, aged between 20 to 30 years. However, the inclusion of infertile patients with different abnormalities (i.e. oligozoospermia, teratozoospermia and asthenozoospermia) categorized into different age groups would bring more attention to the potential clinical applications of short ejaculatory abstinence as a possible modality to improve sperm quality. Second, all the basic semen analysis measurements, including sperm morphology, were performed by CASA. Despite being a powerful tool for the rapid and objective assessment of sperm kinematics and morphometry, CASA systems lack the ability to assess sperm tail characteristics as required by the WHO guidelines. Therefore, the study results regarding sperm morphology should be interpreted with caution, however, no significant difference was observed in sperm morphology between short and long abstinence periods. Furthermore, due to the logistical and financial constraints, the sample sizes used for the determination of the advanced semen parameters were relatively small ($n = 20$). The inclusion of larger sample sizes for these parameters would allow for better generalisation of the findings, especially with regards to the second aim of the study. In addition, for the evaluation of ROS generation, intracellular $O_2^{\cdot-}$ was quantified by means of flow cytometry using DHE as a probe. This technique has been proven to be a highly reproducible and robust method for detecting even minute changes in the sperm intracellular $O_2^{\cdot-}$ levels, thereby, it was certainly appropriate for the purpose of the current study. Despite $O_2^{\cdot-}$ being a major ROS and a common contributor to oxidative stress, it would have been advisable to measure other ROS members such as H_2O_2 and OH^{\cdot} as well as total ROS, both intra- and extracellularly. Measuring only ROS and not total antioxidant capacity (TAC) furthermore provides a rather one sided view on oxidative stress. Therefore, determining a ROS–TAC score or measuring the oxidation-reduction potential would provide a more comprehensive view of the oxidative status of a semen sample.

6.4 Future studies

Considering the above-mentioned limitations together with the findings of the current study opens many avenues to further explore and apply this modality of short ejaculatory abstinence in both laboratory and clinical settings. Subsequently, a few interesting ideas for future research with regards to the effect of abstinence on sperm quality and fertility outcomes will be discussed.

- Upon ejaculation, spermatozoa are terminally differentiated mature cells and any subsequent important functional changes are assumed to be exerted by the biochemical composition of the epididymal and accessory sex glands (i.e. prostate, seminal vesicles) secretions. Seminal plasma is rich in fructose, which along with glucose is one of the preferred energy substrates of sperm, in addition to numerous ions (i.e. Ca^{++} , Zn^+ , Na^+) and micronutrients (i.e. amino acids, citric acid, peptides, proteins) that support the metabolic needs of the sperm (Juyena and Stelletta, 2012).

The role of seminal plasma on the development of sperm functional capacity has been widely studied; however, the extent to which these effects may be influenced by the length of ejaculatory abstinence and the subsequent effect on seminal plasma composition have not been given much attention. Only one study addressed the effect of abstinence period on semen quality in relation to the epididymal and accessory sex gland secretions, and found significant differences in the concentrations of the selected accessory sex gland biomarkers (Elzanaty et al., 2005). However, it is worth mentioning that the concentration of a specific biomolecule in the overall ejaculate may not reflect the actual availability of these molecules per individual spermatozoon, due to variations in the seminal plasma volume/T.S.C. ratios among semen samples. Alternatively, considering the total amount of a specific molecule in the ejaculate, and dividing that value by the T.S.C., would provide a more representative view of the absolute availability of these biomolecules per individual sperm.

Therefore, in order to establish a causal relationship between the length of the ejaculatory abstinence and sperm quality markers investigated in the current study, it would be interesting for

future research to identify variations in the secretions of these glands (per individual sperm) after the two different abstinence periods (4 hours vs. 4 days) and how these variations may reflect on semen quality as a whole. Furthermore, conducting a correlation analysis between selected seminal plasma biomarkers from specific accessory glands (i.e. fructose, citric acid, alpha glucosidase, Ca^{++} , Zn^{+} , etc.) and CASA-derived parameters can assist in establishing a potential cause and effect relationship, which might contribute towards the further elucidation of idiopathic male infertility.

- Semen proteomics have developed tremendously during the last few years owing to the advancement in technology. “The term proteomics has come to encompass the systematic analysis of protein populations with a goal of concurrently identifying, quantifying, and analysing large numbers of proteins in a functional context. As such, the ultimate goal of most proteomic studies is to determine which proteins or groups of proteins are responsible for a specific function or phenotype” (Verrills, 2006). Human seminal plasma is considered an extremely rich source for protein analysis, as it is easily accessible and contains an extremely high protein concentration, ranging from 35 to 55 mg/mL (Gilany et al., 2015). A long list of distinct protein biomarkers has been identified in human seminal plasma; unfortunately, none of these biomarkers has successfully been translated into clinical practice to improve infertility diagnosis. The seminal plasma proteome is not static, but is rather, like many other body fluids, characterized by a highly dynamic range of protein biomarkers. The number and amount of proteins as well as the properties of individual proteins fluctuate over time in response to various physiological and environmental conditions (Milardi et al., 2013). Furthermore, the interaction between proteins and the status of post-translational modifications can both contribute considerably to the complexity and variability of proteomic picture at any given time (Davalieva et al., 2012).

Consequently, it would be interesting for future studies to investigate the time-based changes in the seminal plasma protein profile in the context of ejaculatory abstinence. This could be achieved by selecting samples from normozoospermic men, abstaining sequentially for ≤ 1 day, 2–3 days,

4–5 days, 6–7 days and >7 days. Collecting semen samples from the same individual would be an effective approach to controlling for potential confounding influences, thereby allowing for the ejaculatory period to be the primary intervention. The identification of differentially expressed seminal plasma proteins and their roles in protein interactions and signalling pathways will provide a deeper insight into the mechanisms by which short abstinence may improve semen/sperm quality, as observed in the current study.

- The purpose of sex selection in humans is to avoid the transmission of sex-linked genetic disorders and to achieve a balance in sex composition within the family. Compared to Y chromosome-bearing spermatozoa, the Y chromosome-bearing spermatozoa have been shown to swim faster and more progressively, but display a shorter lifespan and cannot tolerate exposure to acidic environments or oxidative stress for a prolonged period of time (Oyeyipo et al., 2017). In the present study, short periods (4 hours) of abstinence was associated with higher pH levels and antioxidant capacity and concomitantly increased the proportion of progressively and rapidly motile spermatozoa. It would therefore be important to investigate the possible effect of short ejaculatory abstinence on the ratio of the X- and Y-chromosomes bearing spermatozoa, as it may have a direct impact on the sex of the offspring.
- The evidence from this study implicates a role for short abstinence period in the improvement of various conventional and functional semen quality parameters. Although the study was originally designed to target normozoospermic healthy males, future studies should follow up on these findings, incorporating similar designs and targeting patients with abnormality of semen (e.g. oligozoospermia, asthenozoospermia, teratozoospermia). Patient groups should be homogenous in terms of the type and severity of semen abnormality, allowing ejaculatory abstinence to be the primary intervention. It would be interesting to explore whether consecutive ejaculates with short abstinence (i.e. 1 hour, 2 hours, 3 hours, 4 hours etc.) can improve specific sperm abnormalities and if so, identify the most optimal ejaculatory period for a specific abnormality.

- Assisted reproductive technologies (ART) involve a number of procedures, which aim to address fertility problems and improve a couple's chances of conception. Another important direction for future studies is to determine the influence of ejaculatory abstinence on fertilization, pregnancy outcome and live birth rates post IUI, IVF and ICSI. This can be achieved by recruiting couples with male factor infertility attending fertility clinics. Patients can be categorized according to the period of ejaculatory abstinence (4 days vs. 4 hours, or any other abstinence period selected) prior to ART (IUI, IVF or ICSI). Controlling for the influence of ejaculatory abstinence may provide important information for counselling couples with male factor infertility undergoing fertility treatment and might possibly lead to better outcomes.

6.5 Research Outputs

Published article

- **Ayad, B.M.**, Van der Horst, G. and Du Plessis, S.S., 2017. Short abstinence: A potential strategy for the improvement of sperm quality. Middle East Fertility Society Journal. (In press).
- **Ayad, B.M.**, Van der Horst, G. and Du Plessis, S.S., 2018. Revisiting the relationship between ejaculatory abstinence and semen characteristics. International Journal of Fertility & Sterility, 11(4), 238-246.
- **Ayad, B.M.**, Van der Horst, G. and Du Plessis, S.S. Cementing the relationship between conventional and advanced semen parameters. Systems Biology in Reproductive Medicine. UAAN-2017-0224.
- Goss, D., **Ayad, B.M.**, Skosana, B., Van der Horst, G. and Du Plessis, S.S., Human Fertility. THUF-2017-0203.

Published Abstracts

- **Ayad, B.M.**, Van der Horst, G. and Du Plessis, S.S., 2018. Abstinence length and sperm parameters. Andrology, 4(S2), 38-39.

International Conferences contributions

- **Ayad, B.M.**, Van der Horst, G. and Du Plessis, S.S. Effects of abstinence duration on sperm physiology. Physiology 2016. Joint meeting of the American Physiological Society and The Physiological Society. Convention Centre Dublin, Ireland. 27 July 2016.
- Du Plessis S.S., **Ayad B.M.**, and Van der Horst G. Abstinence length and sperm parameters. The 9th Congress of the European Academy of Andrology. De Doelen International Congress Centre, Rotterdam, The Netherlands. 21-23 September 2016.

Local Conference contributions

- **Ayad, B.M.**, Van der Horst G., and Du Plessis S.S. Different abstinence periods alter sperm kinematics. 59th Annual Academic Day of the Faculty of Medicine and Health Sciences, Stellenbosch University. Tygerberg Campus, Cape Town, South Africa. 13 August 2015.

- Du Plessis S.S., **Ayad B.M.**, and Van der Horst G. Alterations in sperm motility, velocity and superoxide levels due to a shorter abstinence period. 44th Annual conference of the Physiology Society of Southern Africa. The River Club, Cape Town, South Africa. 28-31. August 2016.
- Goss, D., **Ayad, B.M.**, Van der Horst, G., Skosana, B.T., and Du Plessis, S.S. (2017). Abstinence period: Impact on seminal citric acid and sperm motility. 45th Annual conference of the Physiology Society of Southern Africa. Groenkloof campus, Pretoria South Africa. 27 to 31 2017.

MSc student co-supervise

- Dale Goss. 2017. Seminal plasma proteomics and metabolomics of semen samples collected from normozoospermic men after long and short abstinence periods.

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Appendix

Linear regression analysis for the prediction of basic and advanced variable of the 2nd ejaculate (dependent variables) from a set of basic semen parameters from the 1st ejaculate (independent variables).

Table A 1. Linear regression summary for the dependent variable: Semen Volume (mL) of the 2nd ejaculate (Y2) R²= 0.62717074, CV-R²=0.56

N=88	b*	Std.Err. of b*	b	Std.Err. of b	t(81)	p-value	# times in best 20
Intercept			1.045454	0.835006	1.25203	0.214162	
Volume (mL)	0.792591	0.071225	0.471176	0.042342	11.12793	0.000000	20
Progress Motility (%)	0.237019	0.102790	0.014869	0.006448	2.30586	0.023676	20
VAP (%)	-0.189599	0.095013	-0.026857	0.013459	-1.99550	0.049350	16
LIN (%)	0.138556	0.070668	0.019322	0.009855	1.96067	0.053355	8
Viability (%)	-0.077704	0.077793	-0.008651	0.008661	-0.99885	0.320842	13
Normal Morphology (%)	-0.070284	0.071154	-0.008833	0.008942	-0.98777	0.326205	11
PH	Excluded						0
Concentration (mill/mL)	Excluded						2
T.S.C (mill/ejaculate)	Excluded						3
Motility (%)	Excluded						0
VCL (µm/s)	Excluded						2
VSL (µm/s)	Excluded						3
STR (%)	Excluded						10
WOB (%)	Excluded						0
ALH (µm)	Excluded						6
BCF (Hz)	Excluded						6

mL = millilitre, VAP = average path velocity, LIN = linearity, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

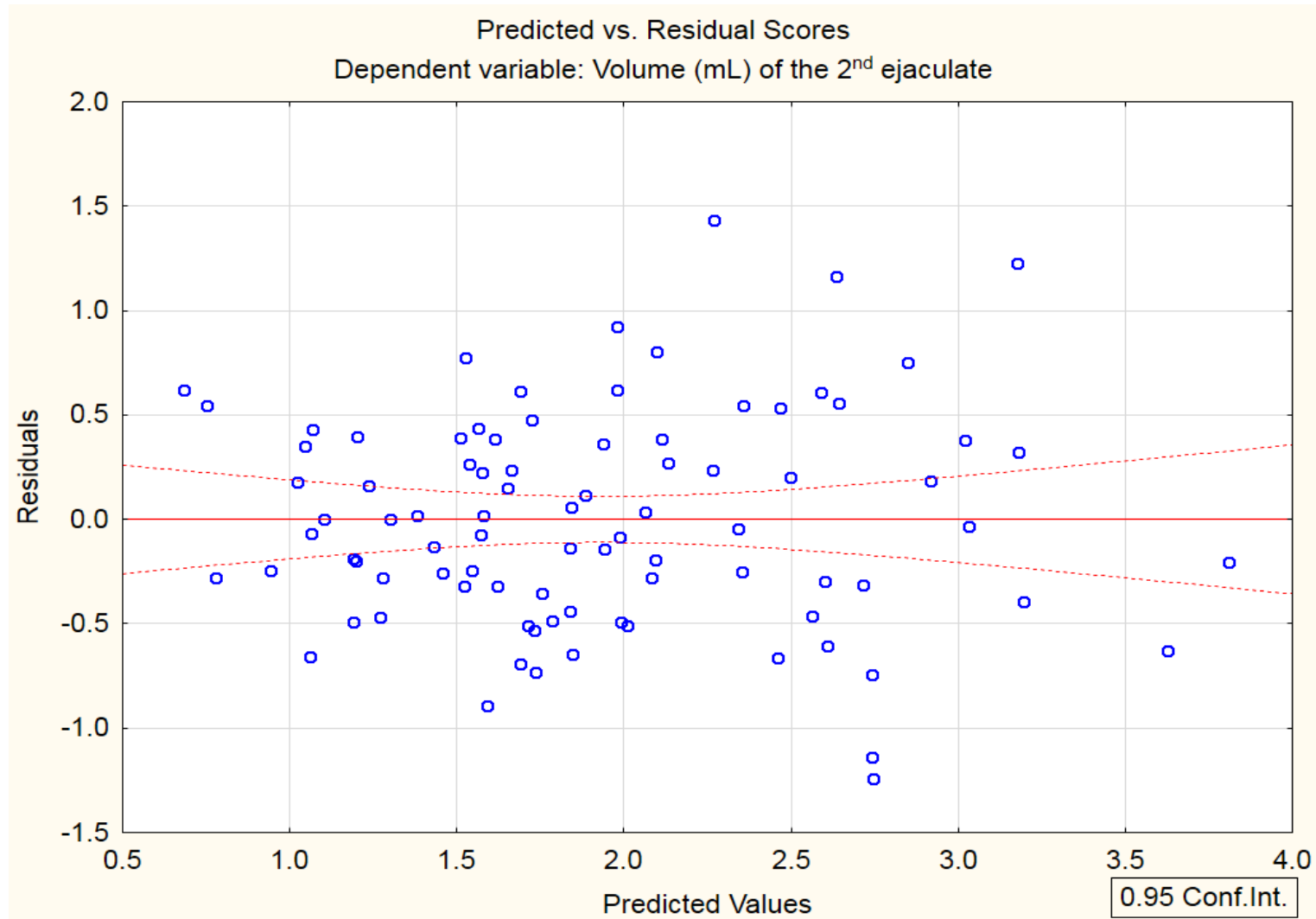


Figure A 1: Regression analysis between predicted variable (semen volume [mL] of the 2nd ejaculate) and the residual scores (a set of basic semen variables). mL = millilitre.

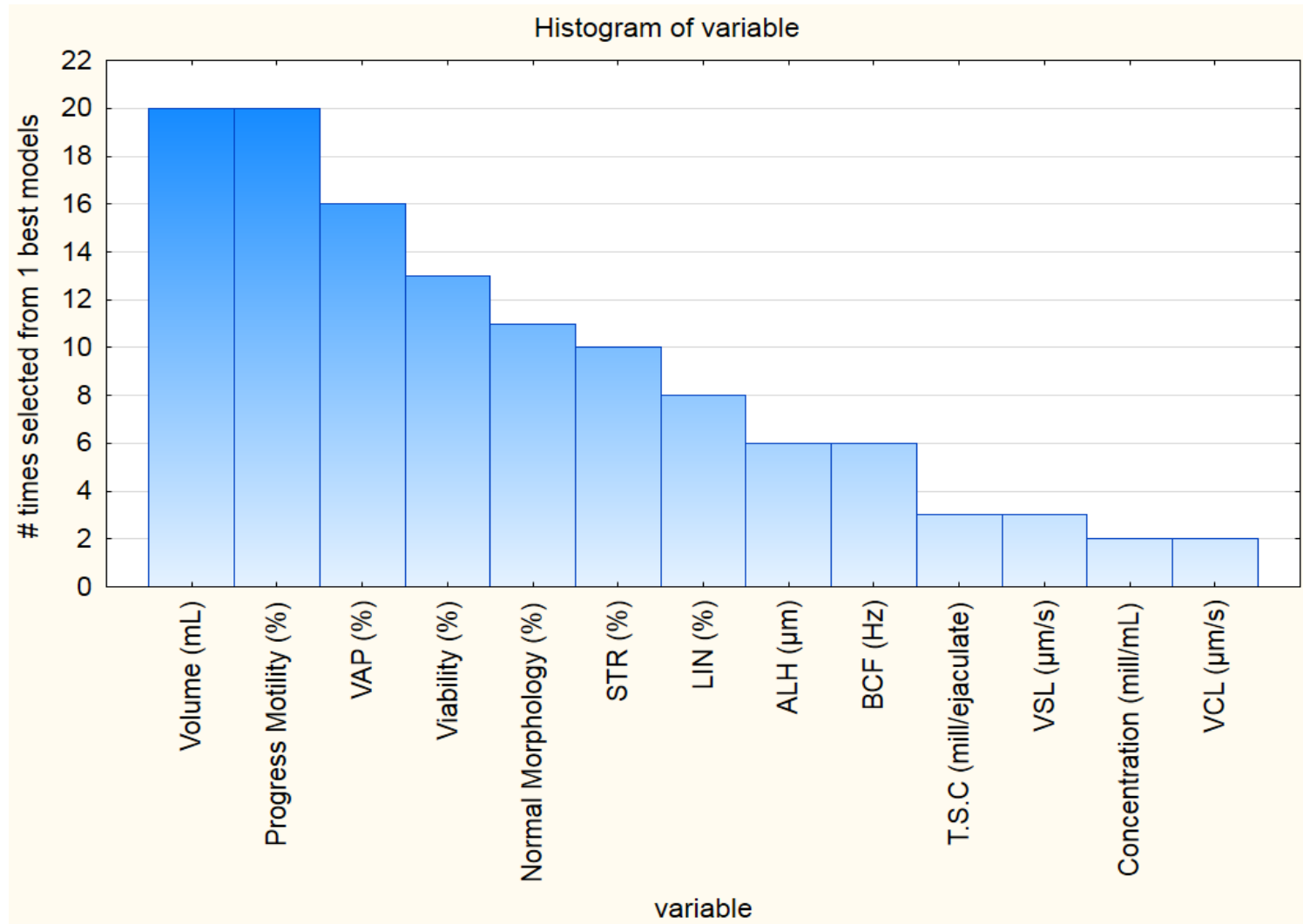


Figure A 2: Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the semen volume (mL) of the second ejaculate as a dependent variable.

Table A 2. Linear regression summary for the dependent variable: Sperm Concentration (10^6 /mL) of the 2nd ejaculate (Y2) $R^2= 0.43940466$, $CV-R^2=0.37$.

N=100	b*	Std.Err. of b*	b	Std.Err. of b	t(95)	p-value	# times in best 20
Intercept			-28.0182	33.67245	-0.83208	0.407450	
Concentration (mill/mL)	0.769233	0.091662	0.4834	0.05760	8.39205	0.000000	19
Progress Motility (%)	0.207614	0.085293	0.3952	0.16236	2.43412	0.016796	7
WOB (%)	0.154649	0.081851	0.9573	0.50668	1.88940	0.061888	5
BCF (Hz)	-0.259354	0.095352	-2.7636	1.01605	-2.71995	0.007764	19
Volume (mL)	Excluded						3
PH	Excluded						3
T.S.C (mill/ejaculate)	Excluded						1
Motility (%)	Excluded						8
VCL (μ m/s)	Excluded						1
VSL (μ m/s)	Excluded						0
VAP (%)	Excluded						5
LIN (%)	Excluded						2
STR (%)	Excluded						1
ALH (μ m)	Excluded						4
Viability (%)	Excluded						0
Normal Morphology (%)	Excluded						2

mL = millilitre, WOB = Wobble, BCF = beat cross frequency, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement. μ m = micrometre, s = second, Hz = hertz.

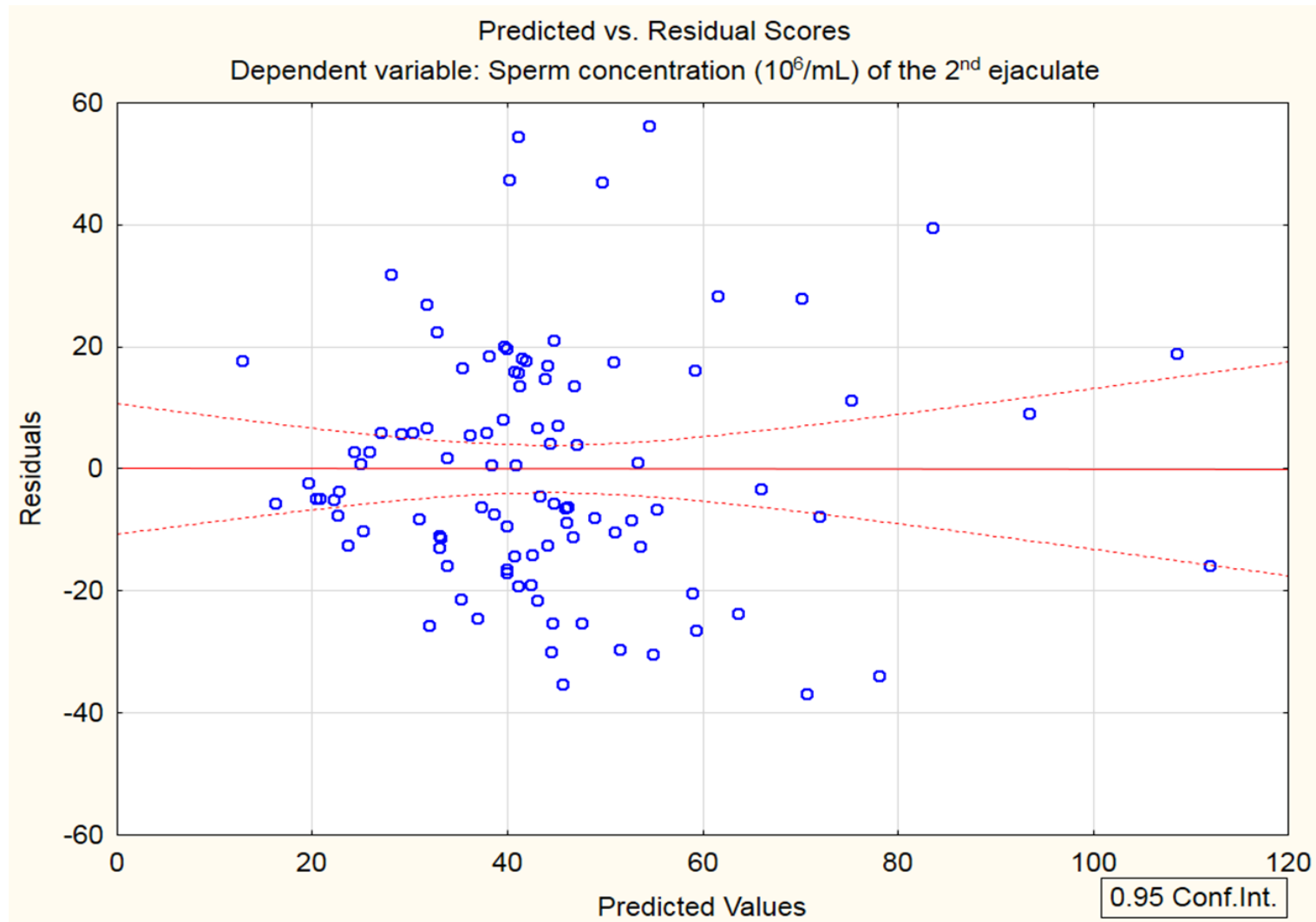


Figure A 3. Regression analysis between predicted variable (sperm concentration [$10^6/\text{mL}$] of the 2nd ejaculate) and the residual scores (a set of basic semen variables). mL = millilitre.

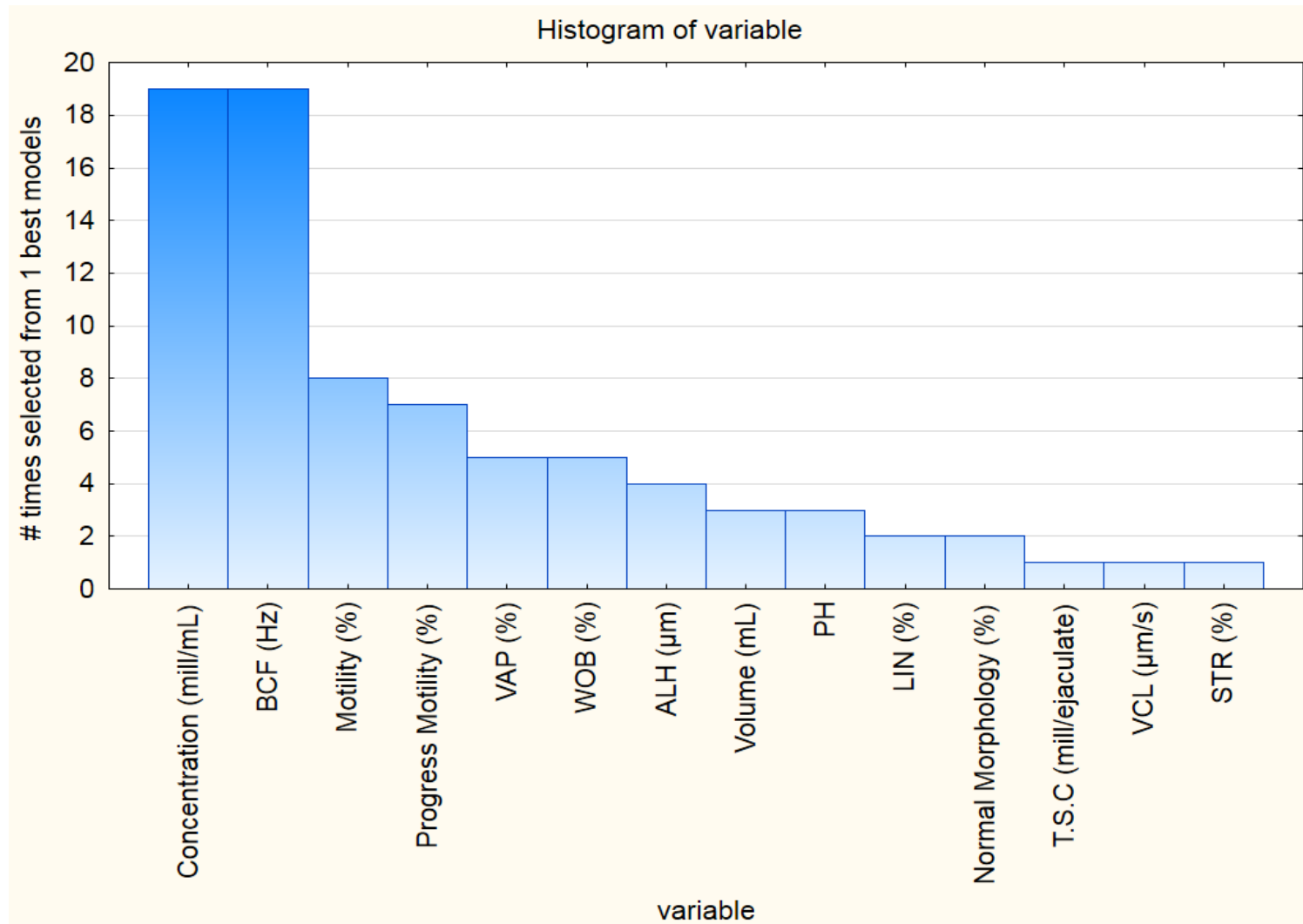


Figure A 4. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm concentration ($10^6/\text{mL}$) of the second ejaculate as a dependent variable.

Table A 3. Linear regression summary for the dependent variable: Sperm Viability (%) of the 2nd ejaculate (Y2) R² = 0.50290624, CV-R²=0.4

N=100	b*	Std.Err. of b*	b	Std.Err. of b	t(97)	p-value	# times in best 20
Intercept			3.538911	7.182462	0.492716	0.623327	
LIN (%)	0.254184	0.073729	0.346569	0.100526	3.447537	0.000838	2
Viability (%)	0.725657	0.073729	0.726906	0.073856	9.842198	0.000000	15
Volume (mL)	Excluded						1
PH	Excluded						1
Concentration (Excluded						1
T.S.C (mill/ejac	Excluded						1
Progress Motilit	Excluded						1
Motility (%)	Excluded						1
VCL (µm/s)	Excluded						1
VSL (µm/s)	Excluded						2
VAP (%)	Excluded						1
STR (%)	Excluded						2
WOB (%)	Excluded						2
ALH (µm)	Excluded						2
BCF (Hz)	Excluded						2
Normal Morpho	Excluded						5

LIN = linearity, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

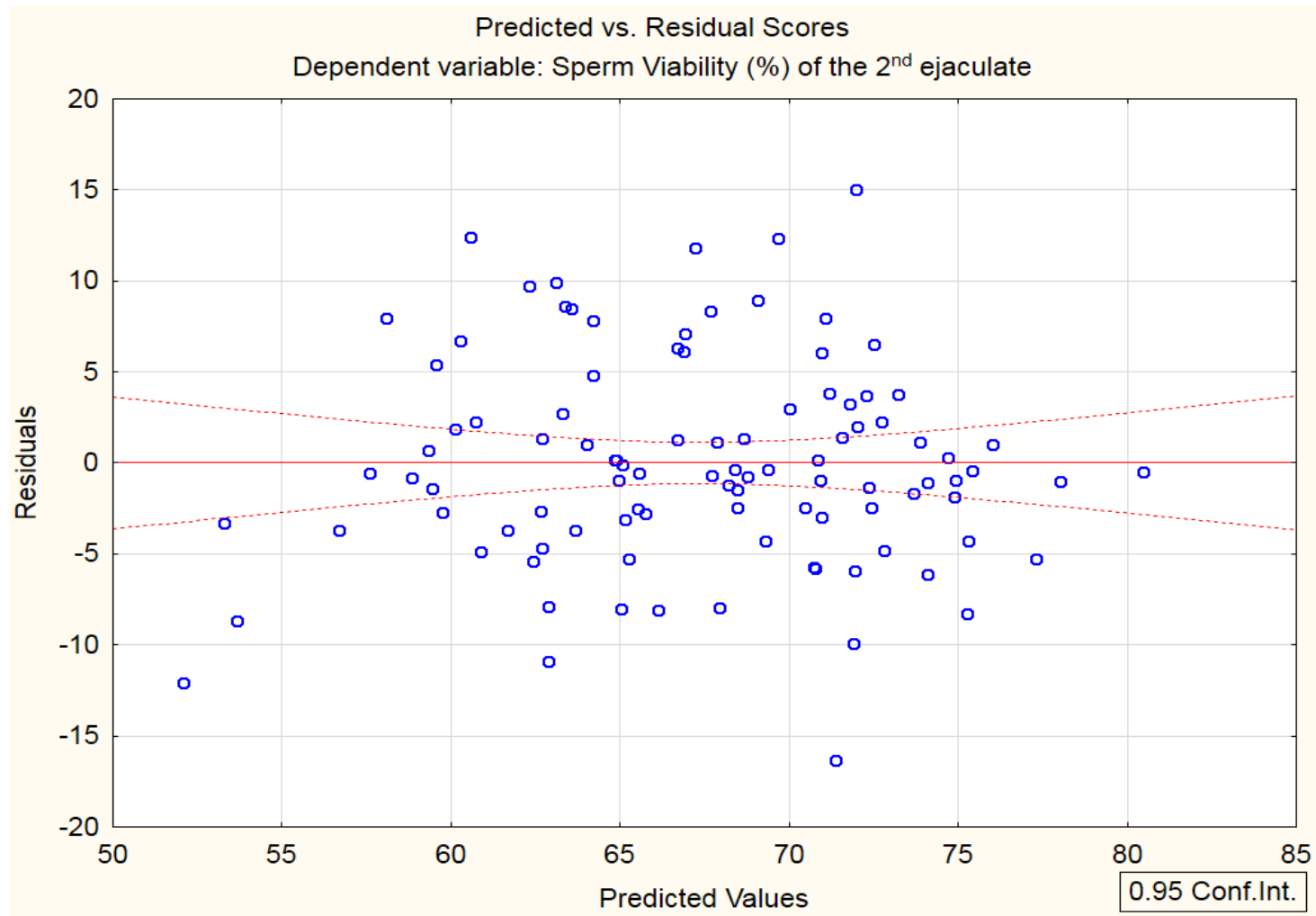


Figure A 5. Regression analysis between predicted variable (sperm viability [%] of the 2nd ejaculate) and the residual scores (a set of basic semen variables).

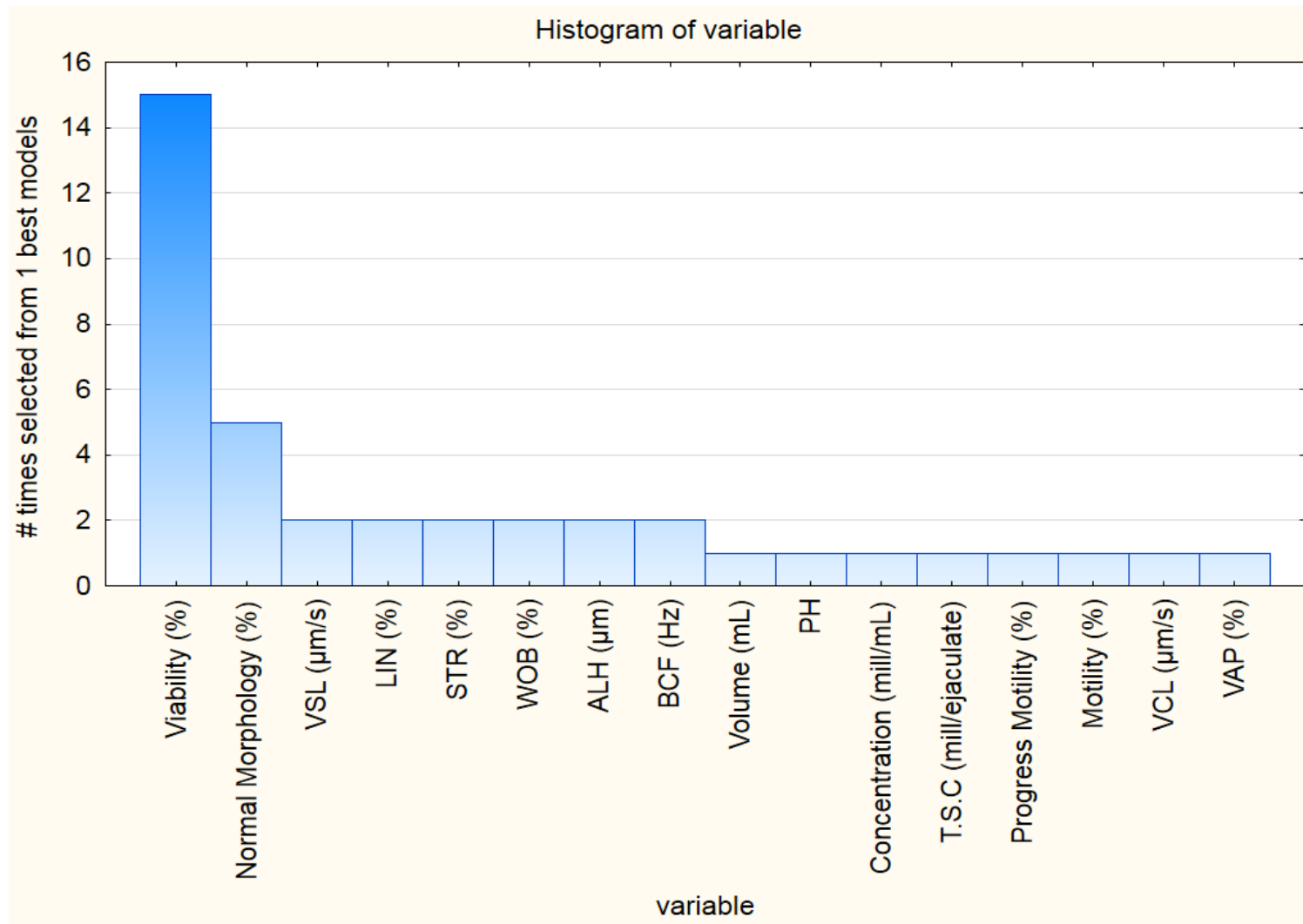


Figure A 6. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm viability (%) of the second ejaculate as a dependent variable.

Table A 4. Linear regression summary for the dependent variable: Sperm Normal Morphology (%) of the 2nd ejaculate (Y2) R²= 0.48290006, CV-R²=0.4

N=88	b*	Std.Err. of b*	b	Std.Err. of b	t(84)	p-value	# times in best 20
Intercept			-4.41804	3.907366	-1.13069	0.261402	
T.S.C (mill/ejaculate)	0.123990	0.078881	0.00480	0.003053	1.57186	0.119742	2
VCL (µm/s)	0.237514	0.079328	0.14371	0.047998	2.99407	0.003615	13
Normal Morphology (%)	0.617343	0.078979	0.59090	0.075596	7.81653	0.000000	20
Volume (mL)	Excluded						1
PH	Excluded						1
Concentration (mill/mL)	Excluded						2
Progress Motility (%)	Excluded						1
Motility (%)	Excluded						1
VSL (µm/s)	Excluded						2
VAP (%)	Excluded						5
LIN (%)	Excluded						3
STR (%)	Excluded						2
WOB (%)	Excluded						2
ALH (µm)	Excluded						1
BCF (Hz)	Excluded						3
Viability (%)	Excluded						1

T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

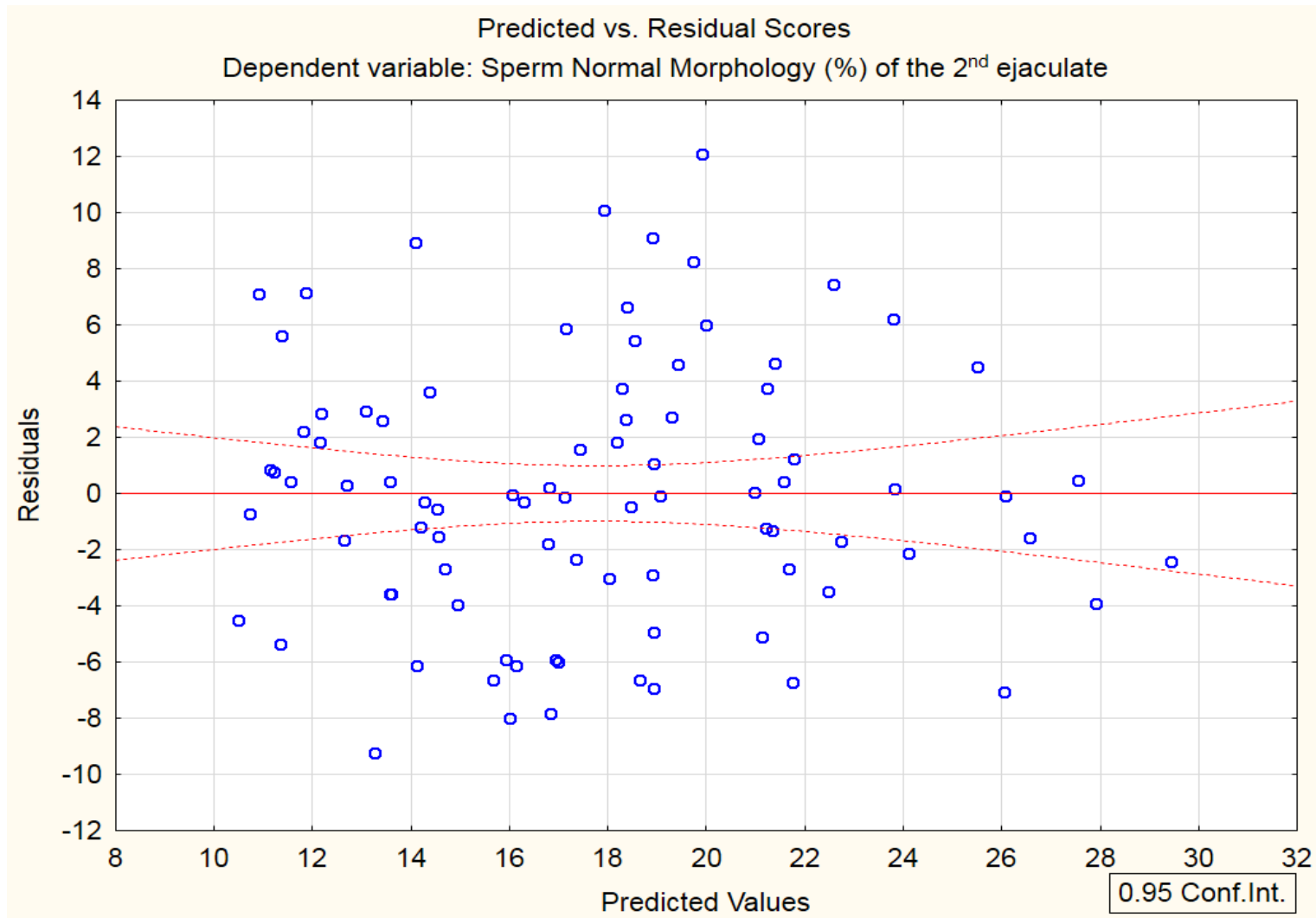


Figure A 7. Regression analysis between predicted variable (sperm normal morphology [%] of the 2nd ejaculate) and the residual scores (a set of basic semen variables).

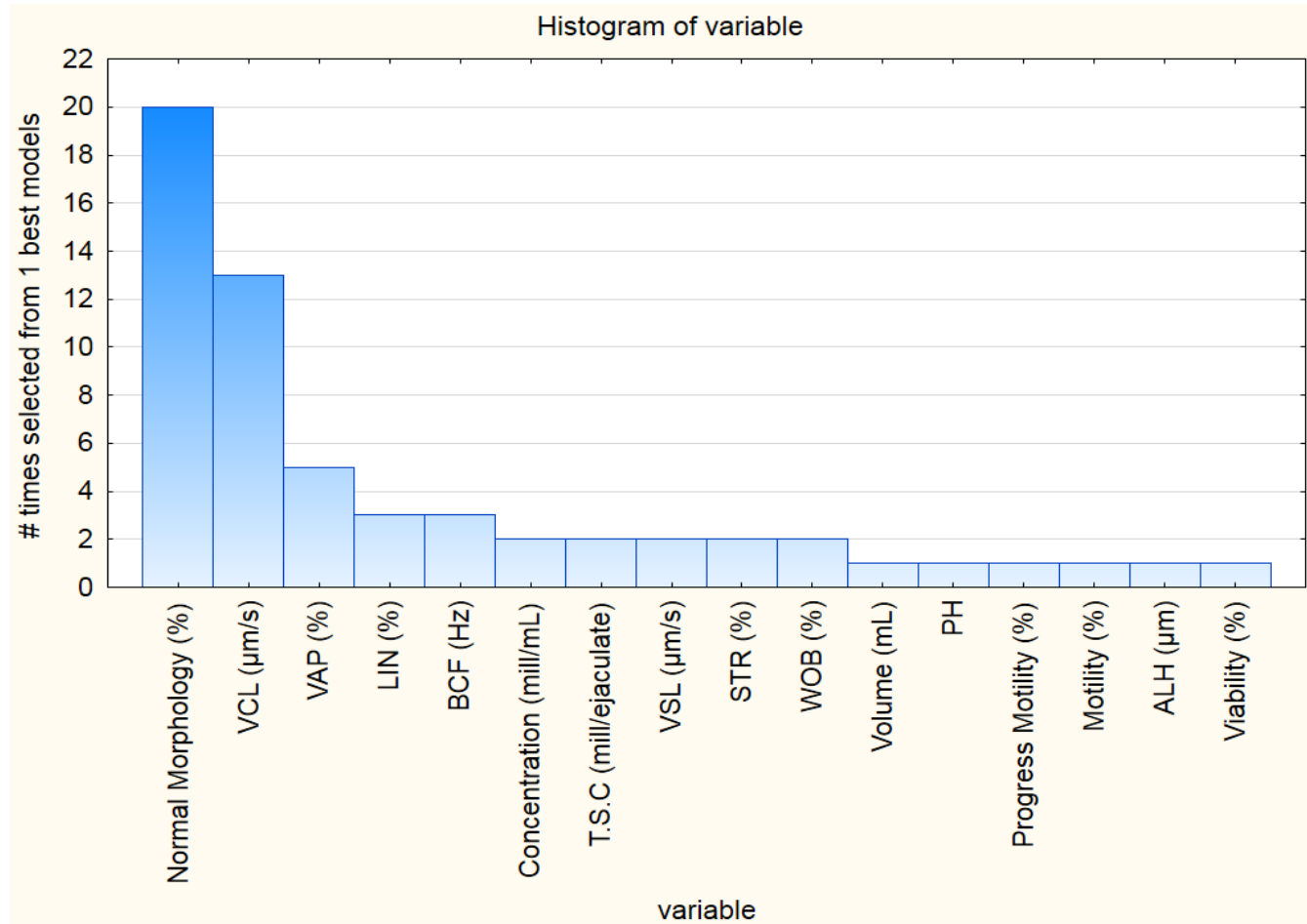


Figure A 8. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm normal morphology (%) of the second ejaculate as a dependent variable.

Table A 5. Linear regression summary for the dependent variable: Sperm Progressive Motility (%) of the 2nd ejaculate (Y2) R²= 0.57582490, CV-R²=0.55.

N=100	b*	Std.Err. of b*	b	Std.Err. of b	t(97)	p-value	# times in best 20
Intercept			3.776743	5.694172	0.66326	0.508734	
Progress Motility (%)	0.702473	0.069347	0.757068	0.074737	10.12977	0.000000	14
ALH (µm)	0.144977	0.069347	6.019380	2.879273	2.09059	0.039179	2
Volume (mL)	Excluded						1
PH	Excluded						2
Concentration (mill/mL)	Excluded						1
T.S.C (mill/ejaculate)	Excluded						1
Motility (%)	Excluded						6
VCL (µm/s)	Excluded						2
VSL (µm/s)	Excluded						1
VAP (%)	Excluded						2
LIN (%)	Excluded						1
STR (%)	Excluded						1
WOB (%)	Excluded						2
BCF (Hz)	Excluded						1
Viability (%)	Excluded						2
Normal Morphology (%)	Excluded						1

ALH = lateral head displacement, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

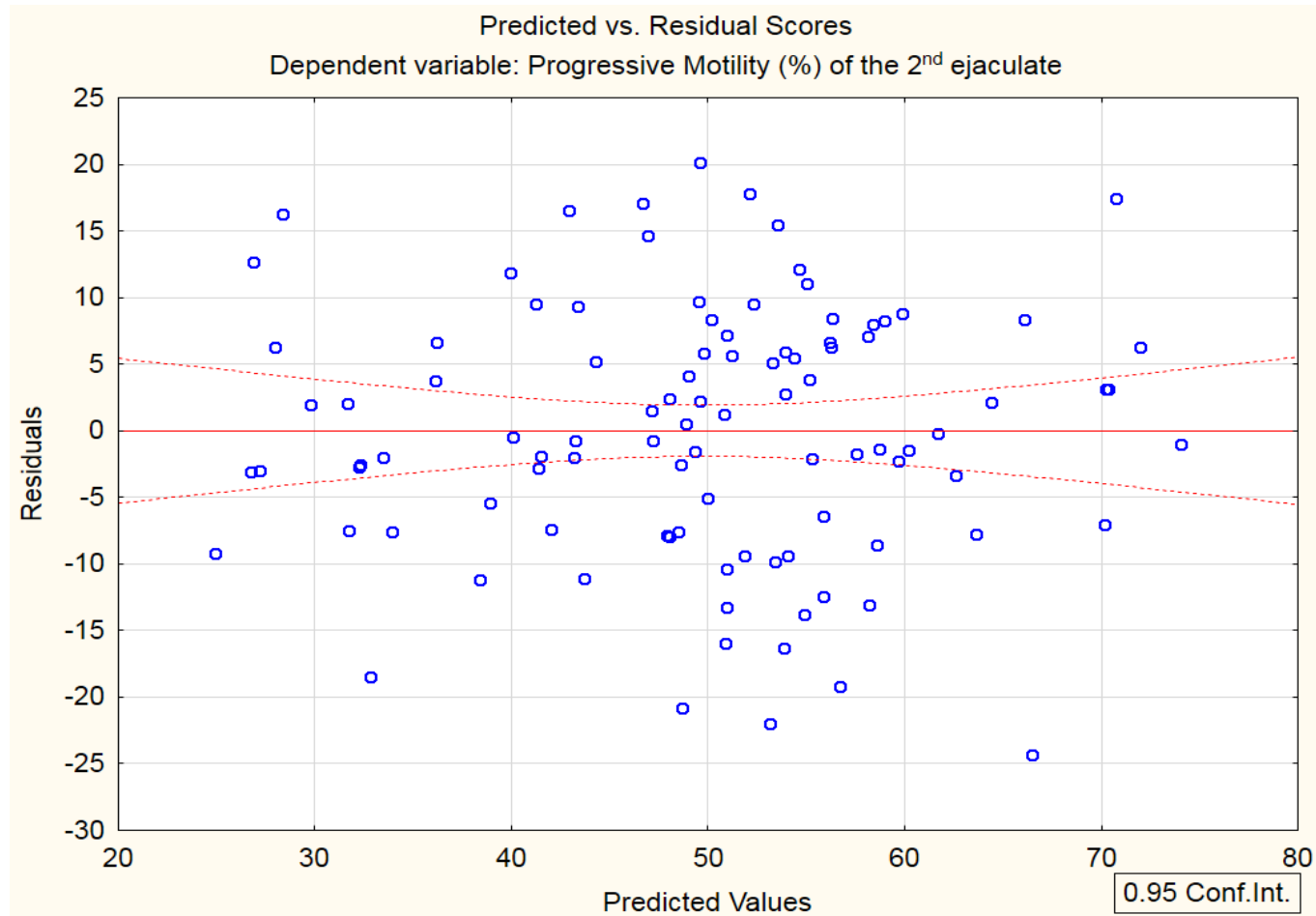


Figure A 9. Regression analysis between predicted variable (Progressive Motility [%] of the 2nd ejaculate) and the residual scores (a set of basic semen variables).

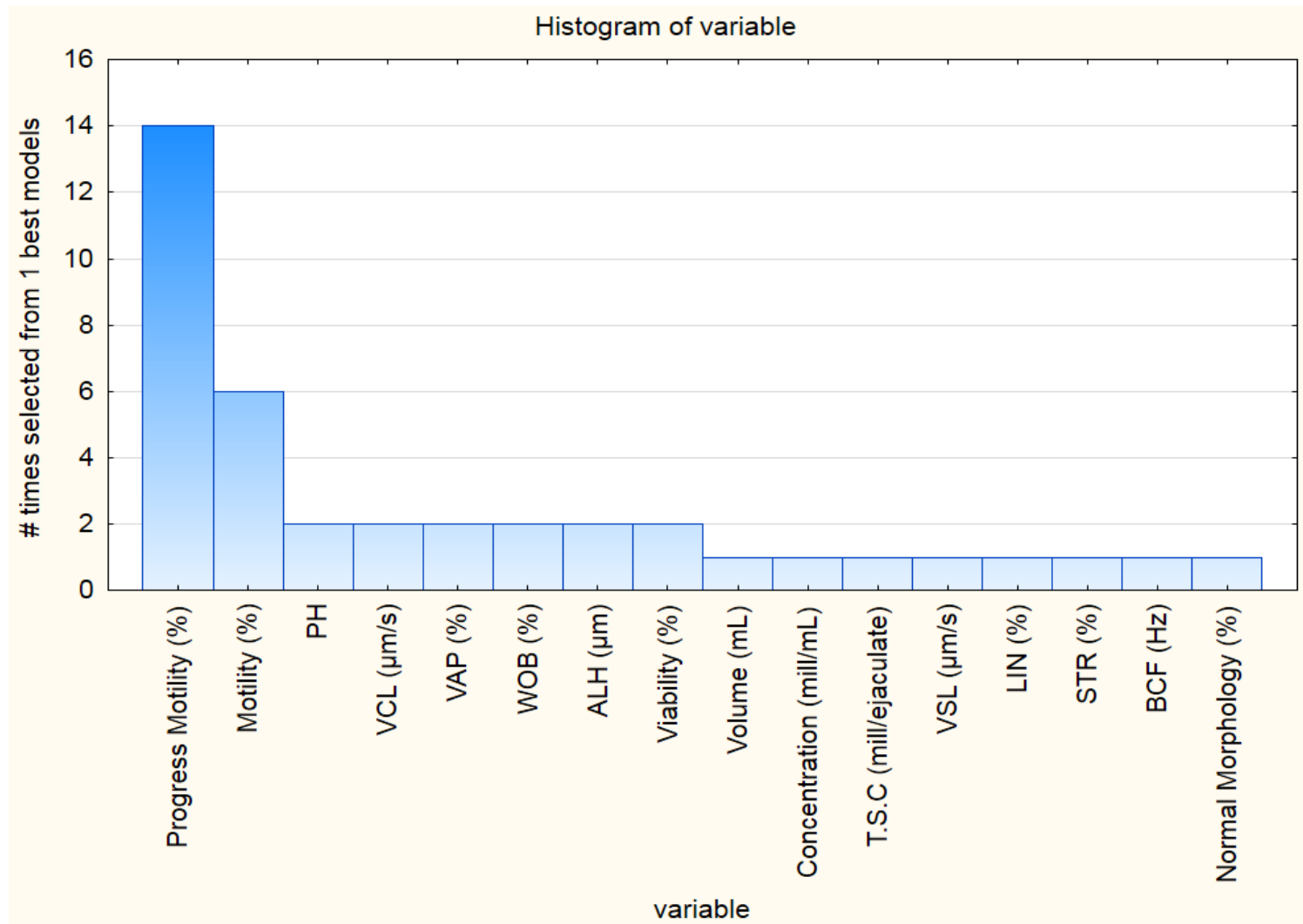


Figure A 10. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of sperm progressive motility (%) of the second ejaculate as a dependent variable.

Table A 6. Linear regression summary for the dependent variable: Sperm Motility (%) of the 2nd ejaculate (Y2) R²= 0.54891289, CV-R²=0.52.

N=100	b*	Std.Err. of b*	b	Std.Err. of b	t(97)	p-value	# times in best 20
Intercept			10.65075	6.378544	1.669778	0.098188	
Motility (%)	0.699637	0.071068	0.71059	0.072181	9.844606	0.000000	14
ALH (µm)	0.116429	0.071068	5.03006	3.070349	1.638271	0.104605	2
Volume (mL)	Excluded						2
PH	Excluded						2
Concentration	Excluded						1
T.S.C (mill/eja)	Excluded						2
Progress Moti	Excluded						6
VCL (µm/s)	Excluded						1
VSL (µm/s)	Excluded						1
VAP (%)	Excluded						1
LIN (%)	Excluded						2
STR (%)	Excluded						1
WOB (%)	Excluded						1
BCF (Hz)	Excluded						1
Viability (%)	Excluded						2
Normal Morph	Excluded						1

ALH = lateral head displacement, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

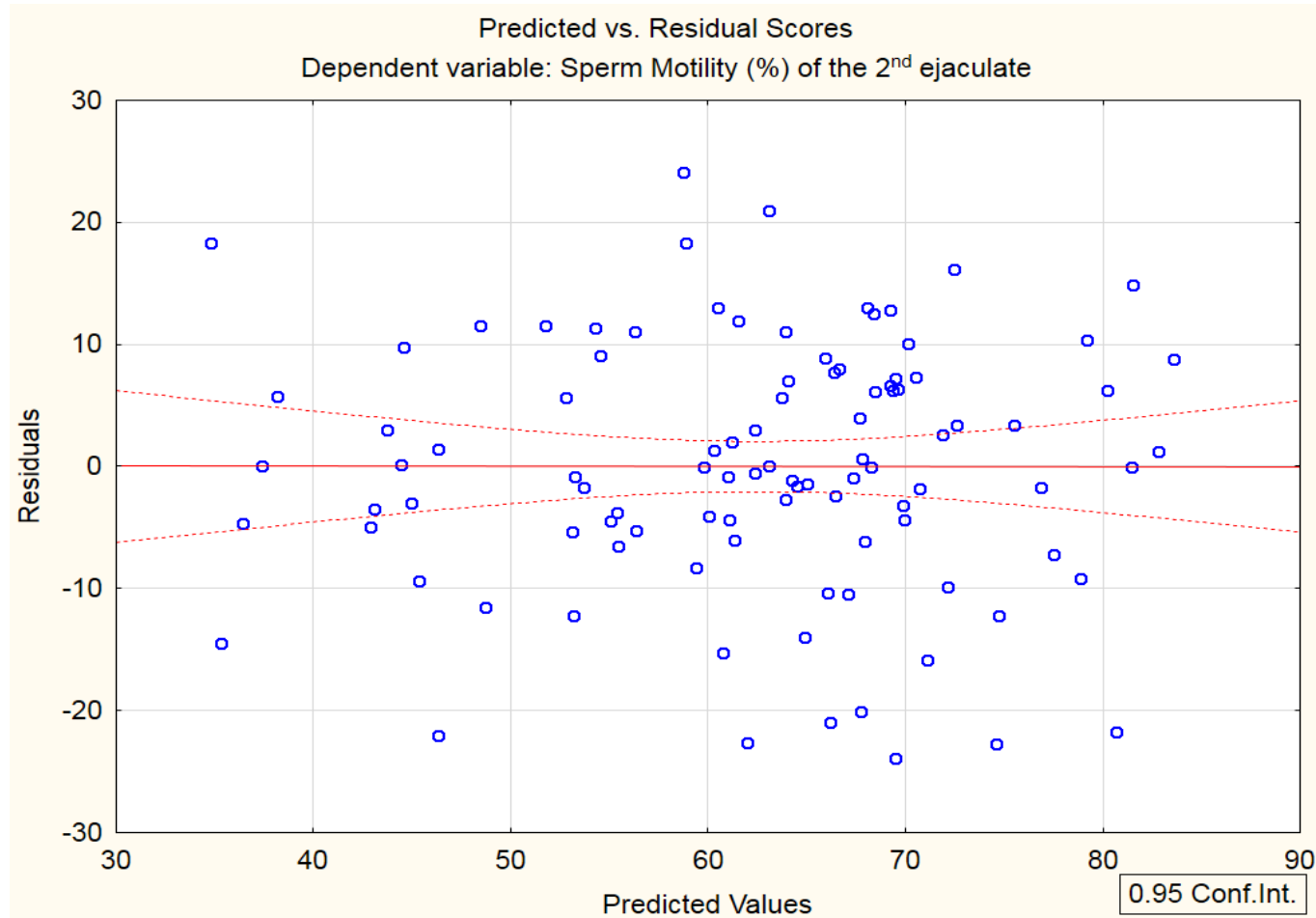


Figure A 11. Regression analysis between predicted variable (Sperm Motility [%] of the 2nd ejaculate) and the residual scores (a set of basic semen variables).

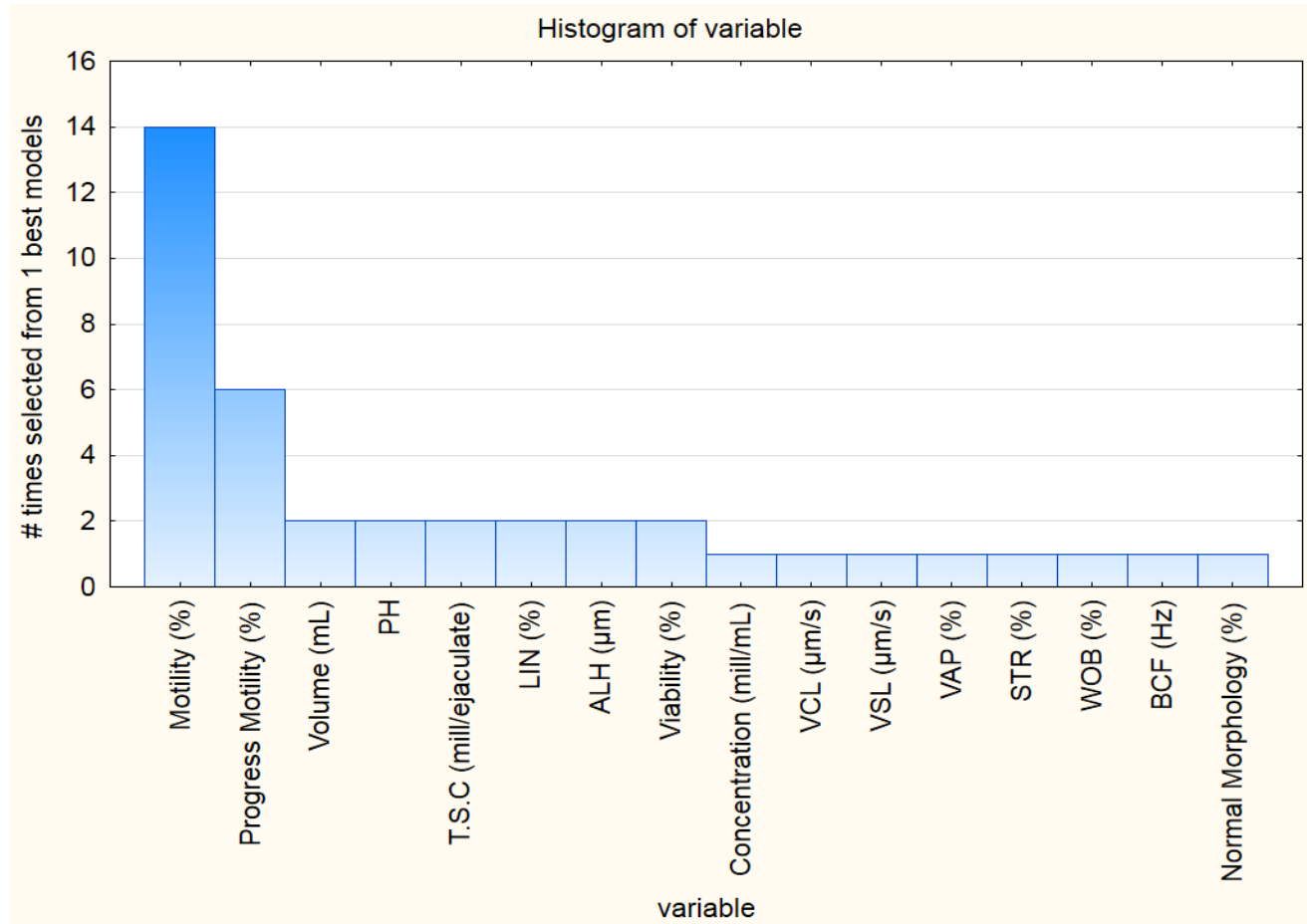


Figure A 12. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm motility (%) of the second ejaculate as a dependent variable.

Table A 7. Linear regression summary for the dependent variable: Sperm DNA Fragmentation (%) of the 2nd ejaculate (Y2) R²= 0.73943379, CV-R²=0.42

N=20	b*	Std.Err. of b*	b	Std.Err. of b	t(14)	p-value	# times in best 20
Intercept			21.9679	8.912924	2.46472	0.027260	
Concentration (mill/mL)	-0.513259	0.235692	-0.1070	0.049118	-2.17767	0.047025	10
T.S.C (mill/ejaculate)	0.597812	0.204586	0.0291	0.009950	2.92206	0.011145	11
Progress Motility (%)	-0.286798	0.149233	-0.1093	0.056874	-1.92182	0.075221	8
ALH (µm)	-0.452650	0.151628	-12.8999	4.321185	-2.98526	0.009835	20
BCF (Hz)	0.808698	0.170710	1.5151	0.319820	4.73727	0.000318	20
Volume (mL)	Excluded						9
PH	Excluded						2
Motility (%)	Excluded						6
VCL (µm/s)	Excluded						1
VSL (µm/s)	Excluded						0
VAP (%)	Excluded						0
LIN (%)	Excluded						4
STR (%)	Excluded						5
WOB (%)	Excluded						0
Viability (%)	Excluded						3
Normal Morphology (%)	Excluded						1

VAP = average path velocity, LIN = linearity, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

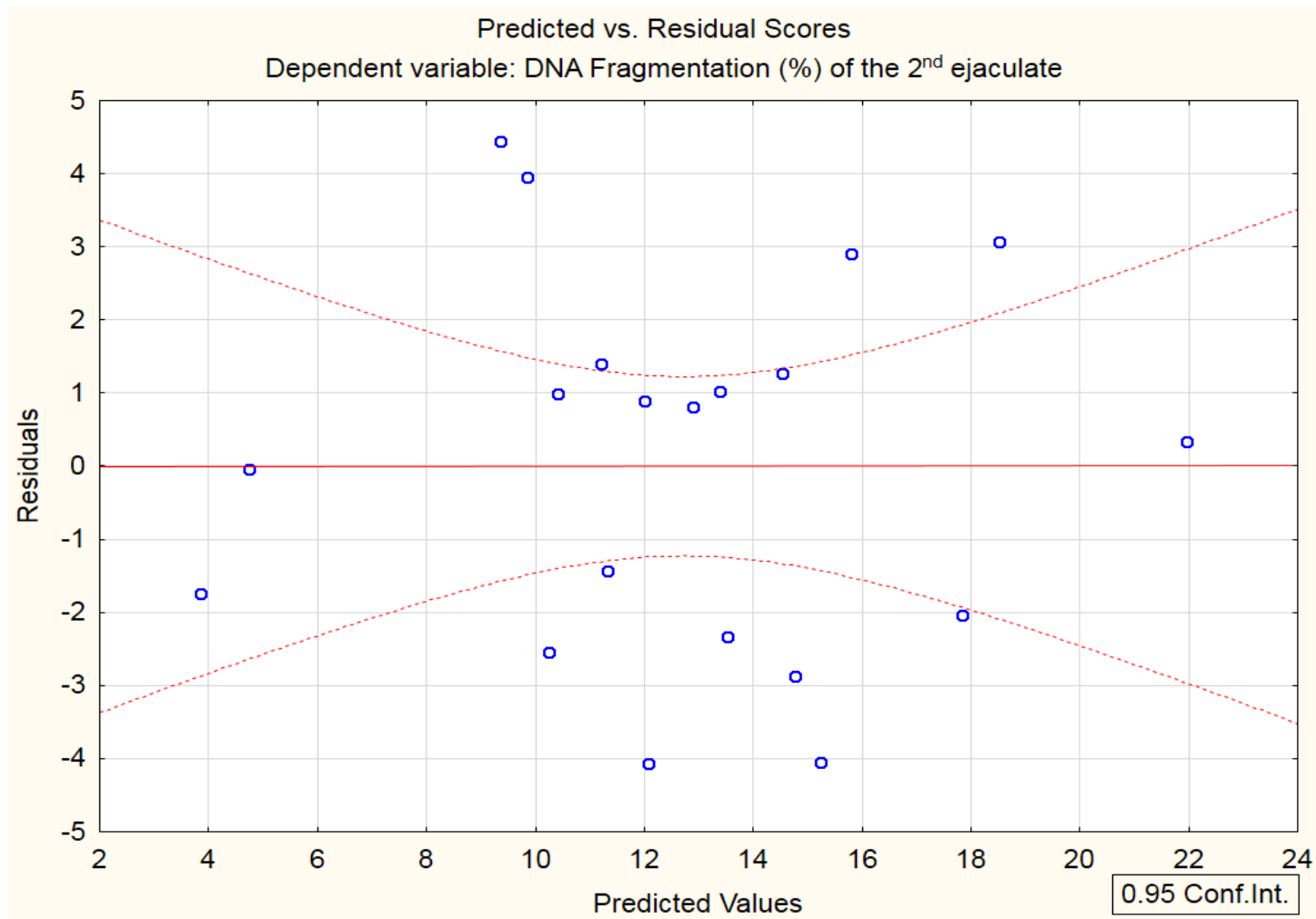


Figure A 13. Regression analysis between predicted variable (DNA fragmentation [%] of the 2nd ejaculate) and the residual scores (a set of basic semen variables).

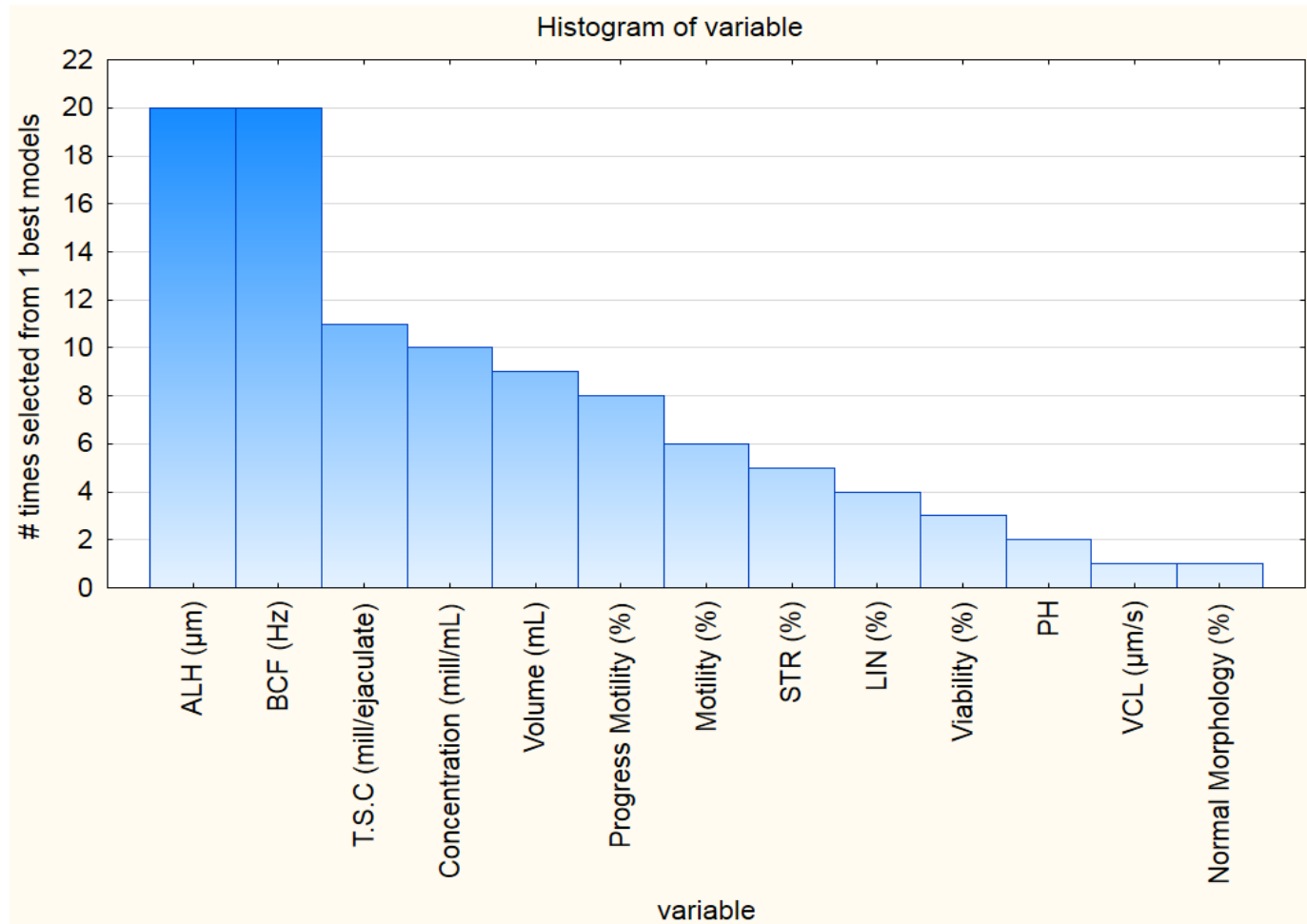


Figure A 14. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm DNA fragmentation (%) of the second ejaculate as a dependent variable.

Table A 8. Linear regression summary for the dependent variable: Sperm Intracellular O₂^{-•} (MFI) of the 2nd ejaculate (Y2) R²= 0.28767301, CV-R²=0.12

N=20	b*	Std.Err. of b*	b	Std.Err. of b	t(16)	p-value	# times in best 20
Intercept			-411.304	429.1692	-0.95837	0.352134	
Volume (mL)	-0.368786	0.217719	-33.252	19.6311	-1.69386	0.109666	20
VSL (µm/s)	-0.312199	0.240874	-5.875	4.5329	-1.29611	0.213324	2
WOB (%)	0.443417	0.234863	13.549	7.1767	1.88798	0.077301	8
PH	Excluded						2
Concentration (mill/mL)	Excluded						1
T.S.C (mill/ejaculate)	Excluded						1
Progress Motility (%)	Excluded						0
Motility (%)	Excluded						1
VCL (µm/s)	Excluded						12
VAP (%)	Excluded						2
LIN (%)	Excluded						1
STR (%)	Excluded						2
ALH (µm)	Excluded						2
BCF (Hz)	Excluded						2
Viability (%)	Excluded						2
Normal Morphology (%)	Excluded						2

O₂^{-•} = superoxide, MFI = median DHE fluorescence intensity, VSL = average path velocity, WOB = Wobble, T.S.C. = total sperm count, VCL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

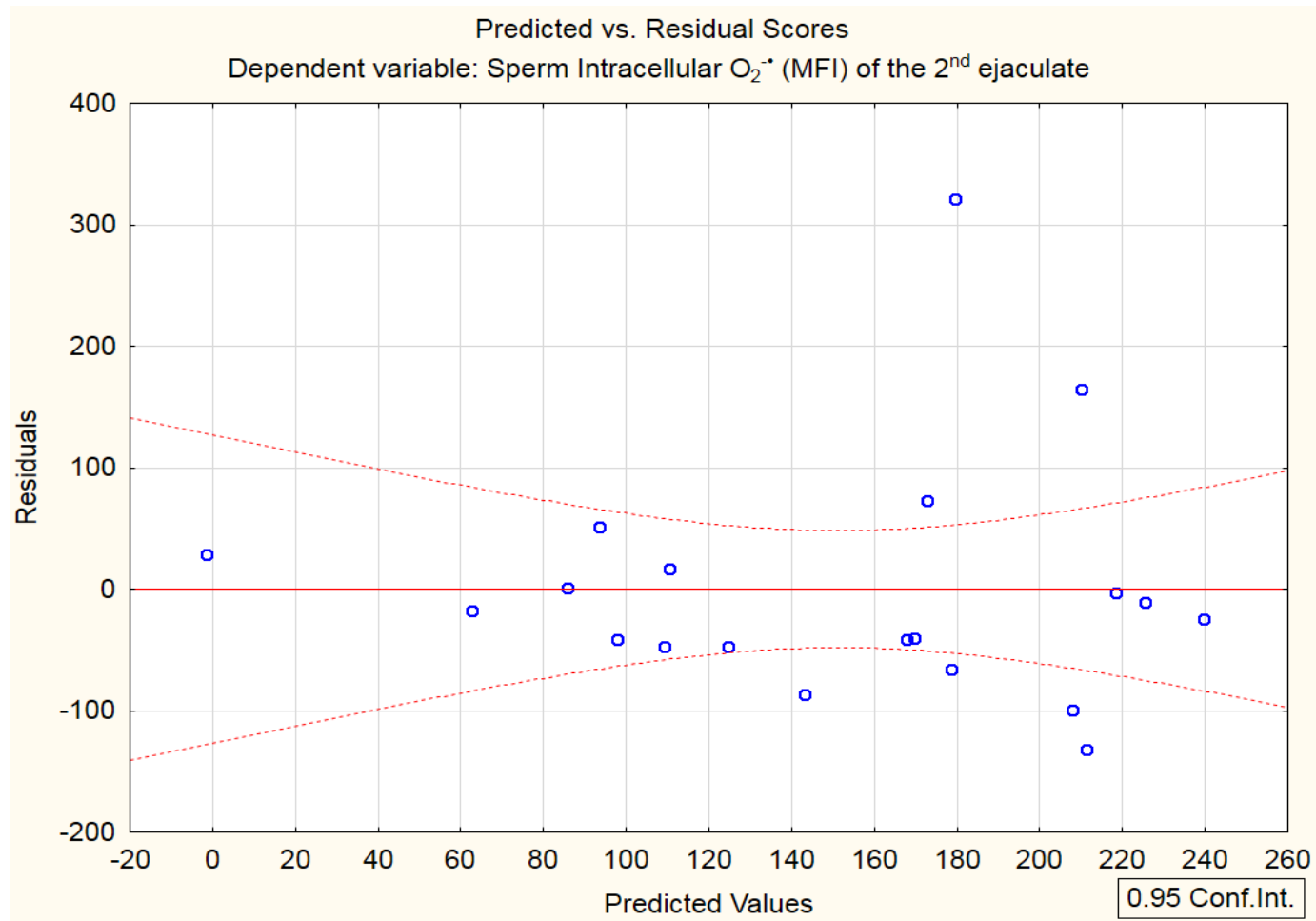


Figure A 15. Regression analysis between predicted (sperm intracellular $O_2^{\cdot-}$ of the 2nd ejaculate) and the residual scores (a set of basic semen variables). $O_2^{\cdot-}$ = sperm superoxide, MFI = median fluorescence intensity.

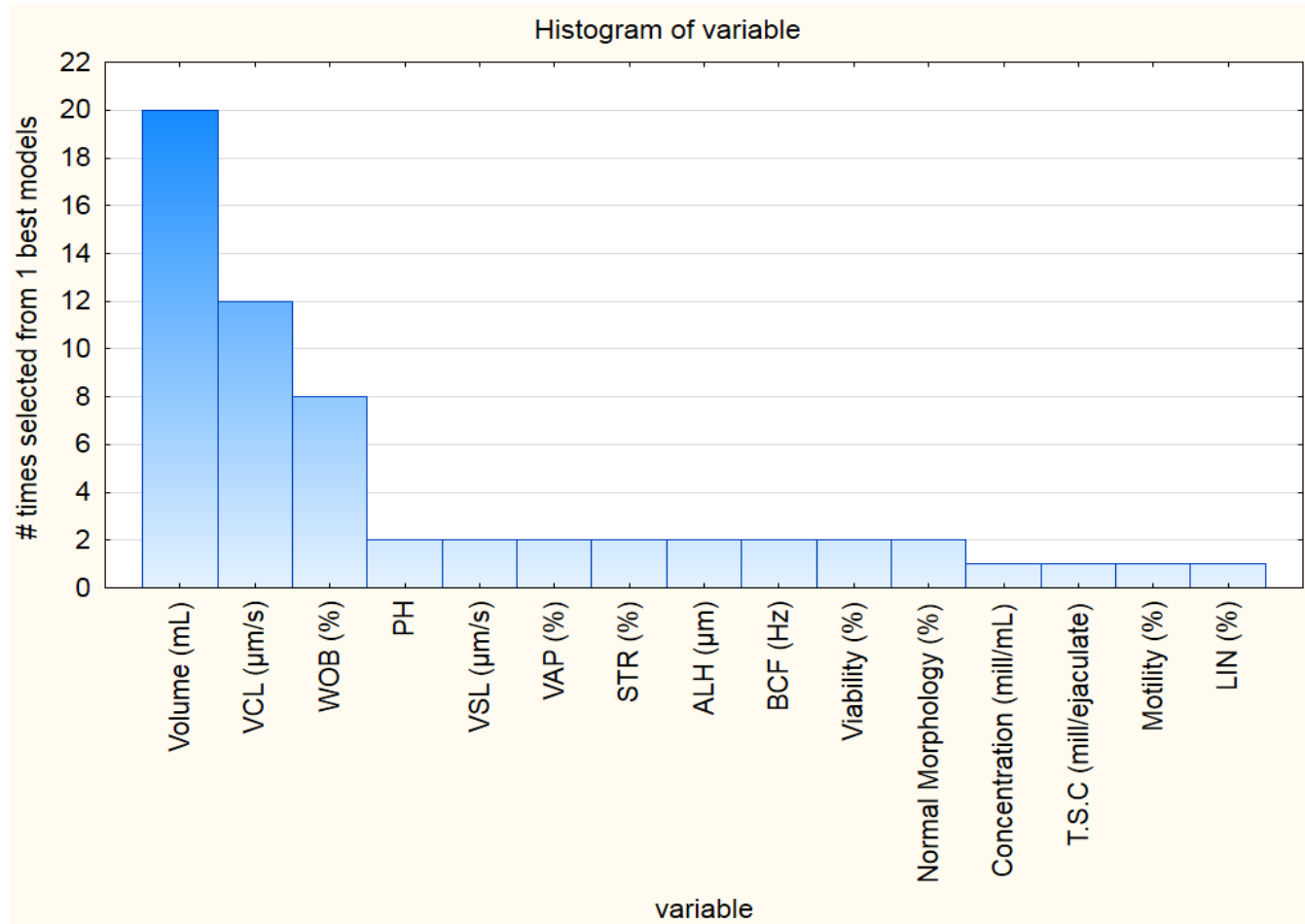


Figure A 16. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the sperm $\text{O}_2^{\cdot-}$ (MFI) of the second ejaculate as a dependent variable. $\text{O}_2^{\cdot-}$ = sperm superoxide, MFI = median fluorescence intensity.

Table A 9. Linear regression summary for the dependent variable: Seminal Plasma TBARS ($\mu\text{mol/L}$) level of the 2nd ejaculate (Y2) $R^2= 0.61361095$, $CV\text{-}R^2=0.33$

N=22	b*	Std.Err. of b*	b	Std.Err. of b	t(16)	p-value	# times in best 20
Intercept			498.7157	145.9141	3.41787	0.003525	
Volume (mL)	-1.12043	0.354940	-12.9705	4.1089	-3.15667	0.006109	16
PH	-0.46051	0.167726	-52.7785	19.2228	-2.74562	0.014363	19
Concentration (mill/mL)	-0.91148	0.386899	-0.5723	0.2429	-2.35587	0.031567	9
T.S.C (mill/ejaculate)	0.95470	0.460406	0.1512	0.0729	2.07360	0.054621	3
Motility (%)	-0.34364	0.177723	-0.4647	0.2404	-1.93355	0.071061	6
Progress Motility (%)	Excluded						5
VCL ($\mu\text{m/s}$)	Excluded						4
VSL ($\mu\text{m/s}$)	Excluded						1
VAP (%)	Excluded						1
LIN (%)	Excluded						1
STR (%)	Excluded						3
WOB (%)	Excluded						2
ALH (μm)	Excluded						13
BCF (Hz)	Excluded						7
Viability (%)	Excluded						9
Normal Morphology (%)	Excluded						1

TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. μm = micrometre, s = second, Hz = hertz.

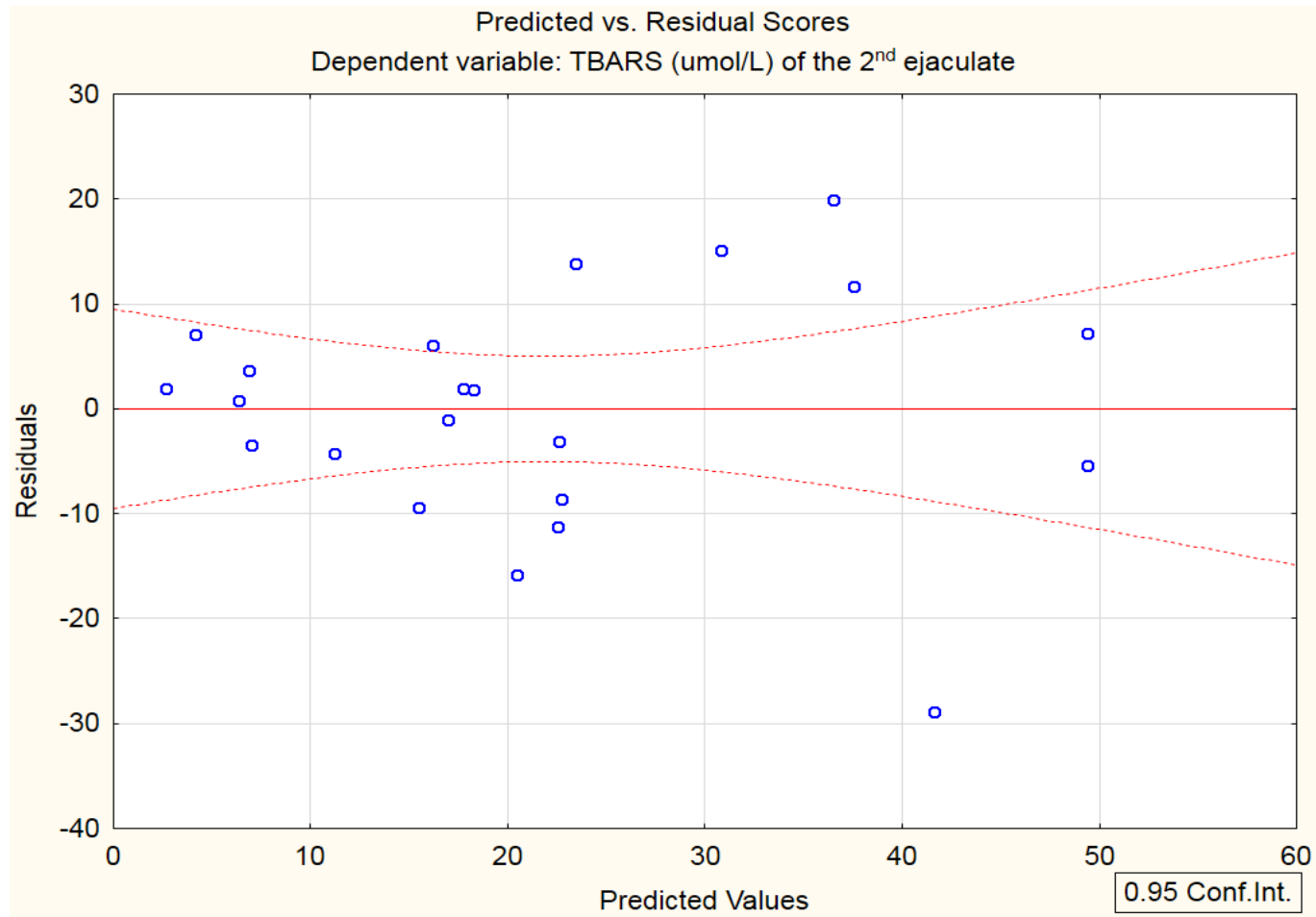


Figure A 17. Regression analysis between predicted (seminal plasma TBARS [$\mu\text{mol/L}$] of the 2nd ejaculate) and the residual scores (a set of basic semen variables). TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre

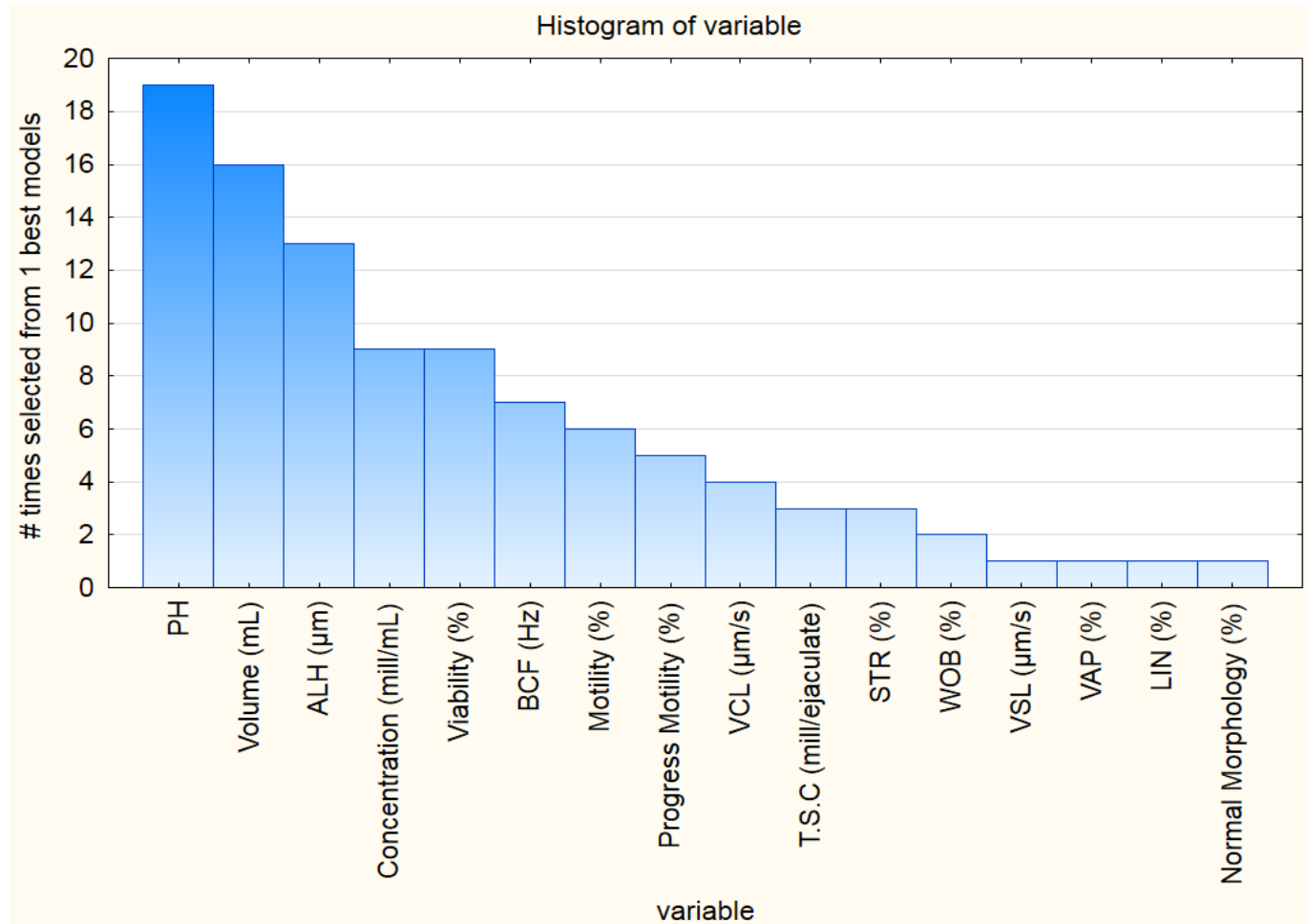


Figure A 18. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma TBARS [$\mu\text{mol/L}$] levels of the second ejaculate as a dependent variable. TBARS = thiobarbituric acid reactive substances, μmol = micromole, L = litre.

Table A 10. Linear regression summary for the dependent variable: Seminal Plasma CAT (U/mL) activity of the 2nd ejaculate (Y2) R²= 0.67005441, CV-R²=0.52

N=19	b*	Std.Err. of b*	b	Std.Err. of b	t(15)	p-value	# times in best 20
Intercept			76.17239	29.07088	2.62023	0.019305	
T.S.C (mill/ejaculate)	-0.453722	0.160360	-0.03492	0.01234	-2.82939	0.012684	14
WOB (%)	-0.269317	0.159547	-0.71390	0.42292	-1.68801	0.112086	2
Normal Morphology (%)	0.744229	0.149281	0.95166	0.19089	4.98542	0.000163	20
Volume (mL)	Excluded						4
PH	Excluded						1
Concentration (mill/mL)	Excluded						2
Progress Motility (%)	Excluded						2
Motility (%)	Excluded						1
VCL (µm/s)	Excluded						1
VSL (µm/s)	Excluded						3
VAP (%)	Excluded						3
LIN (%)	Excluded						1
STR (%)	Excluded						1
ALH (µm)	Excluded						1
BCF (Hz)	Excluded						1
Viability (%)	Excluded						3

U = unite, mL = millilitre, T.S.C. = total sperm count, WOB = Wobble, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

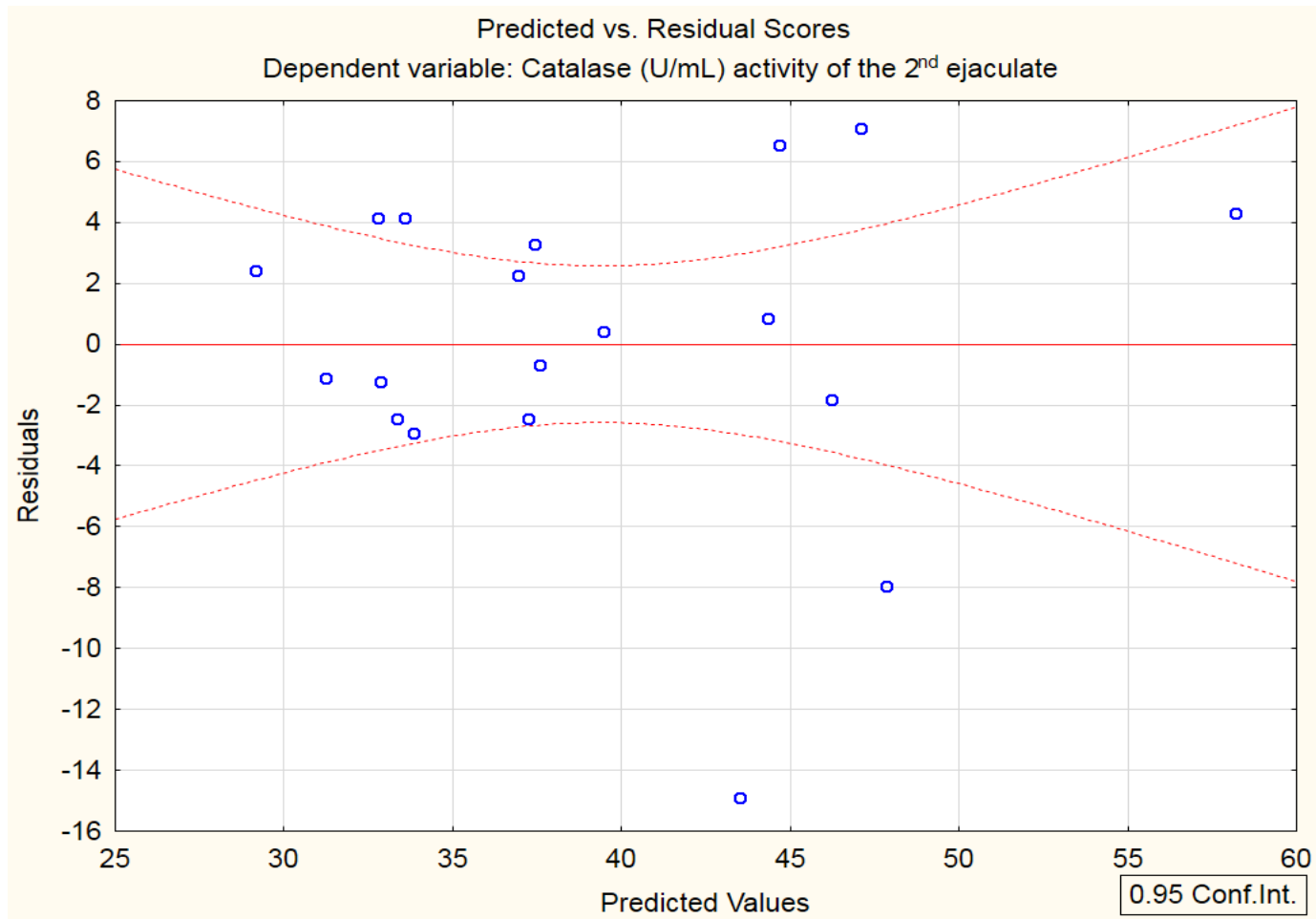


Figure A 19. Regression analysis between predicted (seminal plasma CAT activity [U/mL) of the 2nd ejaculate) and the residual scores (a set of basic semen variables). U = unite, mL = millilitre.

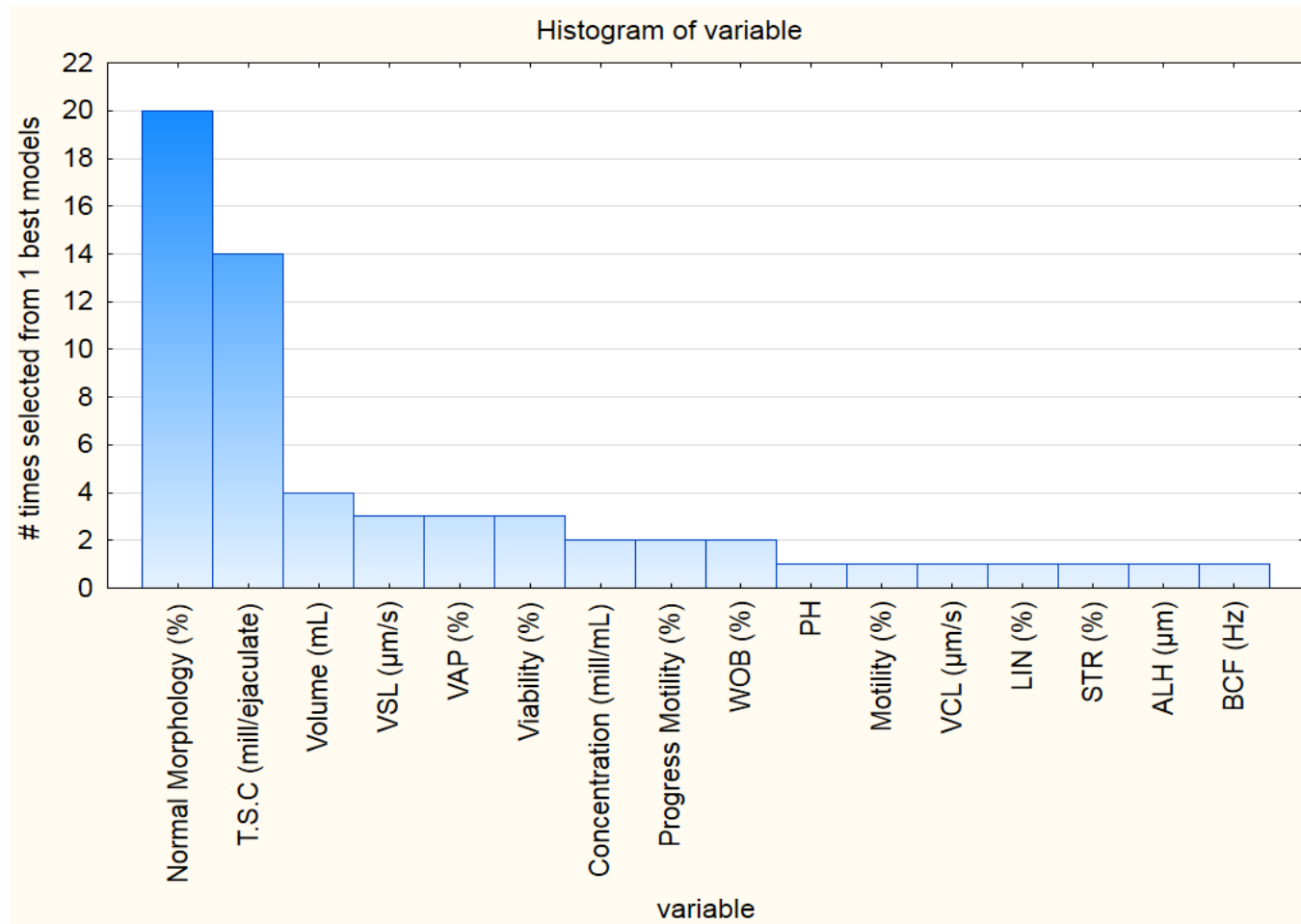


Figure A 20. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma CAT activity (U/mL) of the second ejaculate as a dependent variable. U = unite, mL = millilitre.

Table A 11. Linear regression summary for the dependent variable: Seminal Plasma SOD (U/mg protein) activity of the 2nd ejaculate (Y2) R²= 0.18538541, CV-R²=0.07

N=21	b*	Std.Err. of b*	b	Std.Err. of b	t(16)	p-value	# times in best 20
Intercept			-11.5618	17.72686	-0.65222	0.523520	
PH	0.221724	0.242587	2.1594	2.36259	0.91400	0.374285	18
T.S.C (mill/ejaculate)	0.105152	0.244758	0.0014	0.00326	0.42962	0.673204	5
Progress Motility (%)	-0.421264	0.250890	-0.0539	0.03207	-1.67908	0.112556	8
Viability (%)	0.185074	0.246422	0.0293	0.03905	0.75105	0.463528	18
Volume (mL)	Excluded						2
Concentration (mill/mL)	Excluded						1
Motility (%)	Excluded						10
VCL (µm/s)	Excluded						3
VSL (µm/s)	Excluded						0
VAP (%)	Excluded						2
LIN (%)	Excluded						2
STR (%)	Excluded						4
WOB (%)	Excluded						0
ALH (µm)	Excluded						2
BCF (Hz)	Excluded						3
Normal Morphology (%)	Excluded						2

SOD = superoxide dismutase, U = unite, mg = milligram, mL = millilitre, T.S.C. = total sperm count, VCL = straight-line velocity, VSL = average path velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = Wobble, ALH = lateral head displacement, BCF = beat cross frequency. µm = micrometre, s = second, Hz = hertz.

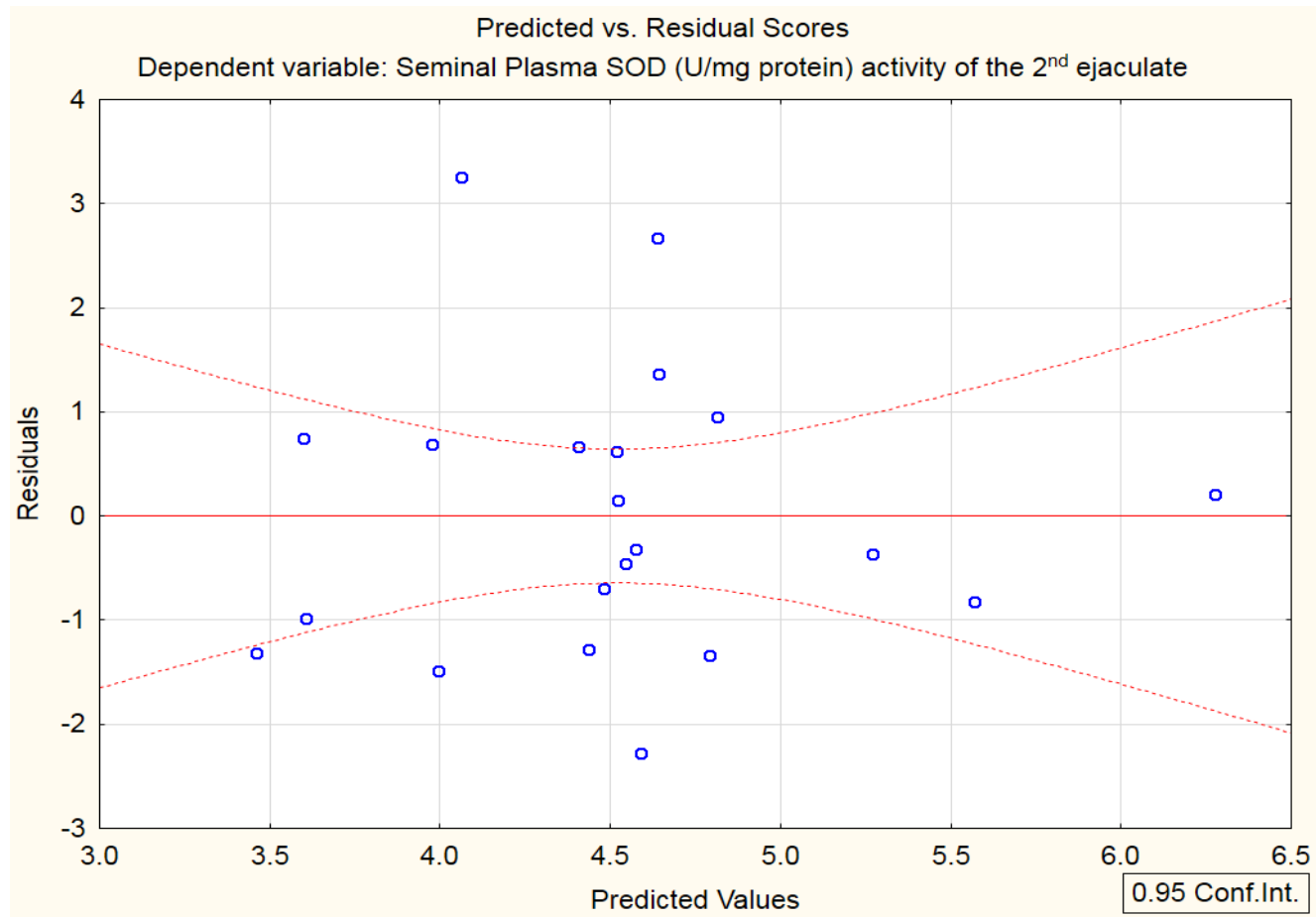


Figure A 21. Regression analysis between predicted (seminal plasma SOD activity [U/mg protein) of the 2nd ejaculate) and the residual scores (a set of basic semen variables). U = unite, mg = milligram.

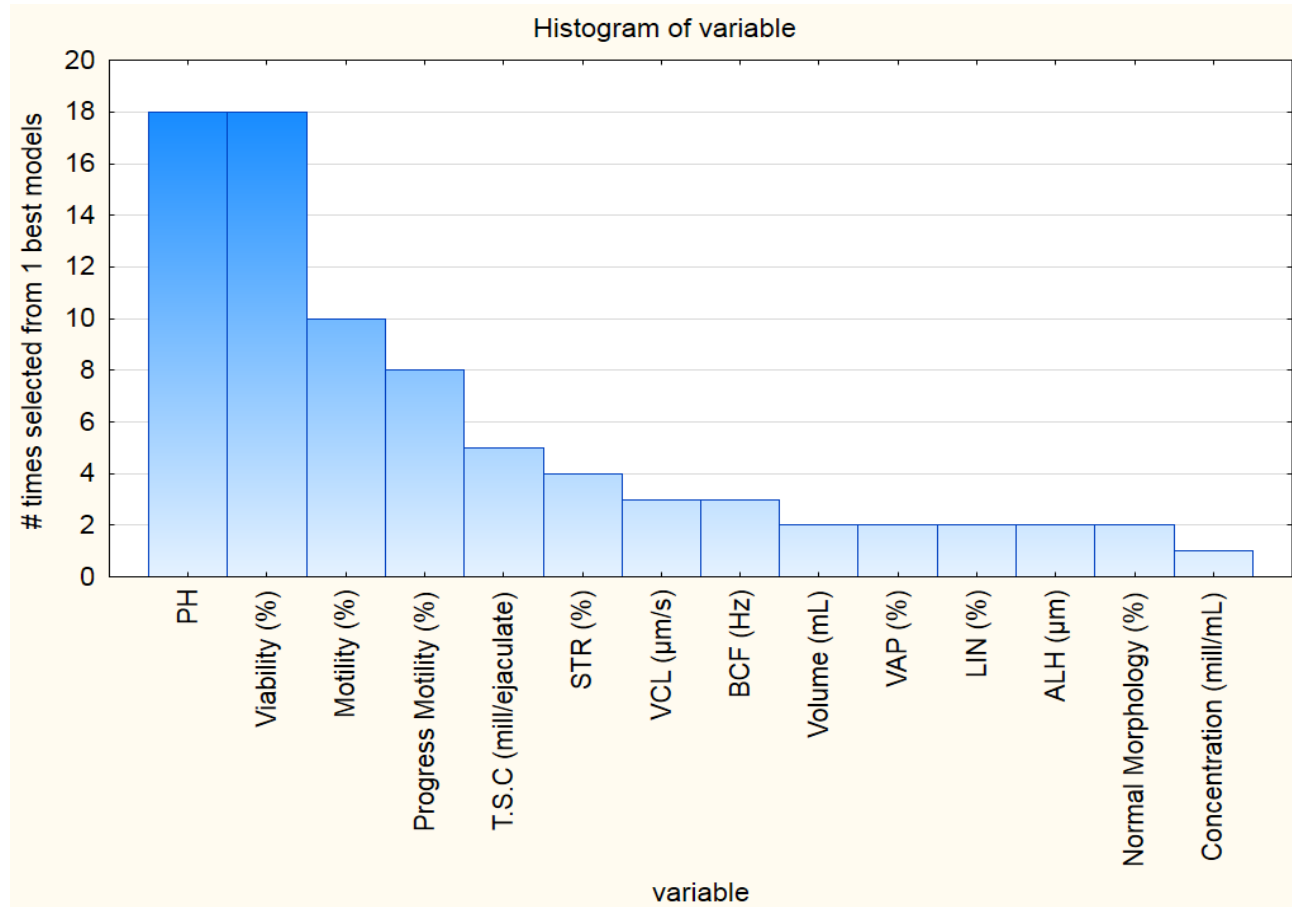


Figure A 22. Histogram representing the distribution of basic semen variables as independent variables, which are considered top in the prediction of the seminal plasma SOD activity (U/mg protein) of the second ejaculate as a dependent variable. U = unite, mg = milligram.