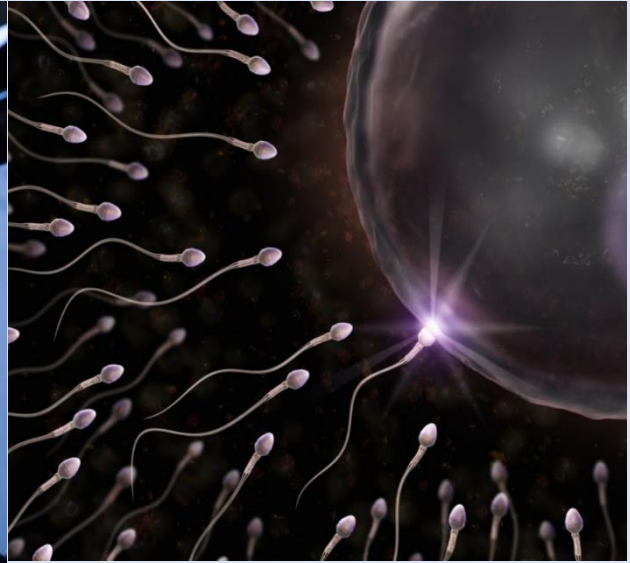
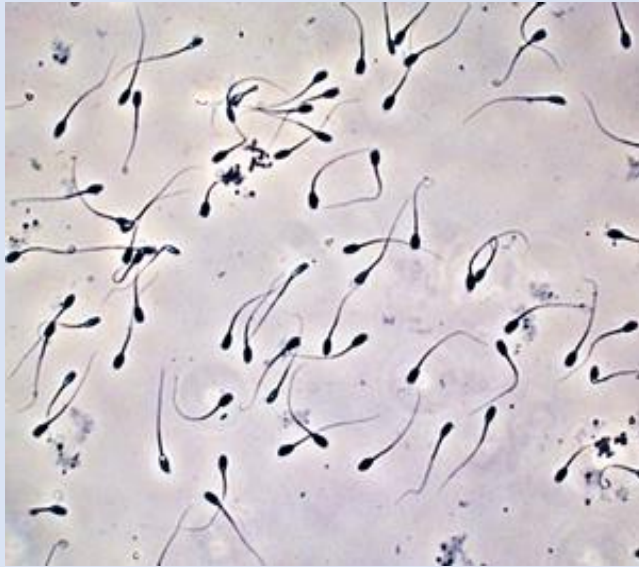


# Validation of the semen analysis method used by the University Medical Center Groningen

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## Abstract

Infertility is a worldwide health problem. The development of assisted reproductive technologies (ART) made it possible for couples suffering from infertility to become pregnant. ART treatments available are intrauterine insemination (IUI), in vitro fertilization (IVF) and intracellular sperm injection (ICSI). Which treatment is advised is dependent on the nature of infertility. Therefore, prior to starting a treatment, men have to undergo a semen analysis to investigate the quality of their semen. The semen analysis according to the World Health Organization (WHO) exists of a macro- and microscopic examination of a fresh semen sample, together with the determination of its spermatozoa concentration and motility. As the ART strategy is partially decided based on the outcome of the semen analysis, it is of importance that the analysis is well validated. Currently, in the UMCG concentration and motility of semen samples is detected via computer-assisted semen analysis (CASA), a computer system prone to technical failure and that is not always accurate with high concentration samples and distinguishing debris from spermatozoa. The analogue alternative is estimating concentration and motility based on observing one field of view under the microscope, a highly inaccurate method. Therefore this project aimed to validate a new analogue counting method, focusing on how many fields of view have to be counted to provide an accurate, though quick estimation. Within this new method it was distinguished whether different objectives and locations caused more accuracy for different concentration groups. Therefore the number of spermatozoa was counted for 10 individual fields of view per sample, alternating between different locations and when possible objectives. The cumulative mean counted spermatozoa was used to determine the estimated sample concentration. The concentration determined via a Neubauer counting chamber was used as reference. Which objective to use and how many fields of view to watch before reaching an acceptable 25% deviance from the Neubauer concentration differs between different semen concentrations. Samples below  $15 \times 10^6$  spermatozoa per ml are best observed with an 20X objective. It then requires three counts to reach the 25% deviance threshold. For samples between 15 and  $30 \times 10^6$  spermatozoa per ml both objectives require five counts to reach the deviance threshold. Using the 40X objective is recommended for these samples considering time investment. Lastly, samples with a concentration higher than  $30 \times 10^6$  spermatozoa per ml can best be observed with a 40X objective. Then it takes only two observations to reach the acceptable deviance, opposing three observations with a 20X objective. Analysis showed that observations done in the inner area and lower half of a counting chamber provided a higher accuracy, though this effect was only seen using a 20X objective. A second part of this project was based around five years of data acquired by the CASA used by the UMCG. The CASA gathers data about semen parameters and this was linked to treatment data such as established fertilization and pregnancies. This data was used to find a possible correlation between the semen parameters and fertilization. A possible correlation was revealed by analysis in *R studio* between percentage of fertilized oocytes and the concentration spermatozoa before sample processing, the average lateral head displacement (ALH) after processing and the total harvested number of oocytes. When looking at pregnancies, a possible correlation was discovered for spermatozoa concentration before sample processing and total percentage of fertilized oocytes. For both possible correlation however, the models did not seem to have a very good fit, so it is recommended to revise the models for more certainty about the correlations.

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## Introduction

### Infertility, numbers and causation

Infertility among couples is a significant health problem worldwide. According to the World Health Organization (WHO) infertility is a failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse<sup>1</sup>. It is estimated that up to 12% of all couples worldwide experience fertility problems<sup>2</sup>. Numbers of 2010 reveal that 1,9% of women between the age of 20 to 44 suffer from primary infertility (inability to attain a live birth), whereas secondary infertility (inability to have another child after at least one live birth) has a frequency of 10,5% among women of reproductive age<sup>3</sup>. Though, numbers of reported infertility are susceptible to the exact definition of infertility<sup>4</sup>.

Causality of infertility is very heterogeneous. It can be caused by female factors, male-factors, a combination of the two but is sometimes unexplained. In females, infertility can be the cause of tubal factors such as bilateral occlusion of the tubes, pelvic adhesions or acquired tubal abnormalities that can be the result of a sexually transmitted infection (STI)<sup>5</sup>. Furthermore, infertility in women can be due to ovulatory disorders, which can have different underlying reasons. Ovulation is strictly regulated through the hypothalamic-pituitary-ovarian (HPO) axis<sup>6</sup>. Ovulatory disorders are mostly caused by either a dysfunction of the hypothalamic-pituitary gland alone or the whole HPO-axis. Sometimes infertility due to ovulatory disorder is caused by failure of the ovarian.<sup>7</sup> In women, more than in men, age also plays a large role in infertility. A higher age in women is strongly associated with higher rates of infertility<sup>8</sup>.

Of all cases of infertility, around 40 to 50 % of them is contributed by a male factor<sup>9</sup>. When there is male-factor infertility, the most common cause is the presence of abnormalities in the composition of their semen<sup>5</sup>. Different abnormalities those men can suffer from are amongst others azoospermia (no spermatozoa in semen), oligozoospermia (too little spermatozoa in semen), asthenozoospermia (decreased motility of the spermatozoa) and teratozoospermia (increased number of spermatozoa with abnormal morphology), or a combination of these abnormalities<sup>10</sup>. These abnormalities can be the result of different underlying problems. For example, azoospermia may be caused by an obstruction of the ejaculatory duct or the vas deferens, the ducts transporting spermatozoa from the epididymis to the ejaculatory duct.<sup>11</sup> When there is no sign of an obstruction, the lack of spermatozoa might be caused by impaired spermatogenesis. Impaired spermatogenesis has various underlying causes such as hypogonadotropic hypogonadism and genetic or congenital abnormalities.<sup>12,13</sup> Obstruction of the seminal vesicles results in a lowered pH of the ejaculate, which decreases motility of spermatozoa<sup>14,15</sup>. Moreover, overall health condition also appears to play a significant role in fertility. Medical conditions such as, but not limited to, chronic obstructive pulmonary disease, cystic fibrosis and multiple sclerosis are found to affect semen parameters negatively<sup>16</sup>. Similarly, most likely through substantial changes in hormone levels due to excess adipose tissue, obesity is associated with infertility in males.<sup>16</sup> The hypothesis that overall health influences fertility is nicely demonstrated by an Italian study that found a correlation between an increasing Charlson Comorbidity Index(CCI), which is a marker of poor overall health, and infertility<sup>17</sup>. Additionally, infertility in men can be the result of other external factors such as previous injuries, (STI) infections or exposure to toxins<sup>7</sup>. Even though for the majority of couples the origin of their infertility can be retraced, for 15-30% of all couples suffering from infertility no apparent cause can be found<sup>18</sup>.

### ART techniques

Over the years, assisted reproductive technologies (ART) have increasingly developed and made it possible for people that suffer from fertility problems to fulfill their wish to conceive. Couples have a variety of different treatments that can be offered depending on their situation. The least invasive ART

is called intrauterine insemination (IUI). IUI is a first-line therapy that is mostly used for patients that have unexplained fertility problems or suffer from infertility caused by cervical factors, immunological factors, moderate male factors or ejaculation disorders.<sup>19</sup> An IUI cycle is less invasive than other therapies because it only requires the collection of a fresh semen sample of the man. The semen is then processed so that the most motile sperm cells are left. This processed semen is then directly inseminated in the uterus.<sup>20</sup> IUI is also performed in combination with controlled ovarian stimulation, ensuring ovulation around the time of insemination.

For couples where IUI treatment is unsuccessful, more invasive treatments are available. *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) can be the next step when none of the previous IUI cycles lead to a pregnancy. Direct referral to IVF can be beneficial for cases with advanced age of the female ( $\geq 35$  y), infertility by tubal pathology or with severe endometriosis.<sup>19</sup> Furthermore, IVF is advised with mild male factor infertility. With IVF, fertilization is attempted by adding a certain amount of spermatozoa per oocyte to oocytes that are prepared in a petridish<sup>21</sup>. The exact number of added spermatozoa is variable among laboratories, as they all have their own protocol. For ICSI, a spermatozoon is directly injected into the cytoplasm of an oocyte<sup>22</sup>. This makes it suitable for couples suffering from severe male factor infertility such as extreme low sperm concentrations or diminished motility.<sup>23</sup>

Both techniques require collection of one or more mature oocytes. Oocytes can be found in the ovaries in complexes called follicles, which are made up of the oocytes surrounded by granulosa cells<sup>24</sup>. Currently two different methods are used to ripen one or more follicles. With controlled ovarian hyperstimulation (COH), the woman gets administered a mixture of gonadotropins and gonadotropin-releasing hormone (GnRH) analogues. This combination of hormones leads to the maturation of multiple follicles.<sup>25</sup> With COH however, comes the risk of developing ovarian hyperstimulation syndrome (OHSS). With modified natural cycle (MNC) IVF, the risk for OHSS is minimized as this method is mainly based on the natural menstrual cycle of the woman. Administration of a GnRH antagonist prevents a premature LH-surge. Subsequently, gonadotropins are administered to support growth and maturation of the follicle. Around 36 hours before collection of the follicle, an LH-trigger, nowadays often in the form of a human choriongonadotrophin (hCG) injection, is given to induce ovulation.<sup>26</sup> The MNC method will typically yield one mature follicle per cycle. Via transvaginal oocyte retrieval the mature follicles are then collected from the ovaries just before the ovulation would take place in natural conditions<sup>21,27</sup>.

### Semen analysis

Prior to starting an ART-trajectory, men have to undergo a semen analysis to assess the quality of their semen. Most fertility labs use the semen analysis as described by the World Health Organization (WHO)<sup>28,29</sup>. This semen analysis is performed on a fresh semen sample that is collected by masturbation after preferably 2-7 days of abstinence. The analysis exists of a macroscopic analysis of the sample (volume, color, odor, liquefaction and viscosity), pH-determination, and a microscopic analysis (agglutination due to presence of anti-sperm antibodies and presence of other cells than spermatozoa). The fresh, undiluted sample is used to determine motility and after dilution the concentration is established. If necessary the sample will also be judged for vitality (in the case of low motility) and morphology.

Performing the semen analysis is essential for determining the strategy for an ART-treatment. The analysis can uncover if there is a male-factor fertility problem. When male-factor infertility is detected, the semen analysis can give more clarification about the nature of the infertility (azoospermia, asthenozoospermia etc.). The nature of infertility is leading in the choice for an ART-strategy. Furthermore, the semen analysis is of clinical importance too. When performing IVF, semen analysis is



used to determine the spermatozoa-concentration on the day of the treatment. This is necessary to establish the volume needed to add sufficient sperm-cells to the ovum. Moreover, semen analysis is important in ICSI-treatments to assure the spermatozoa have sufficient motility on the day of the treatment.

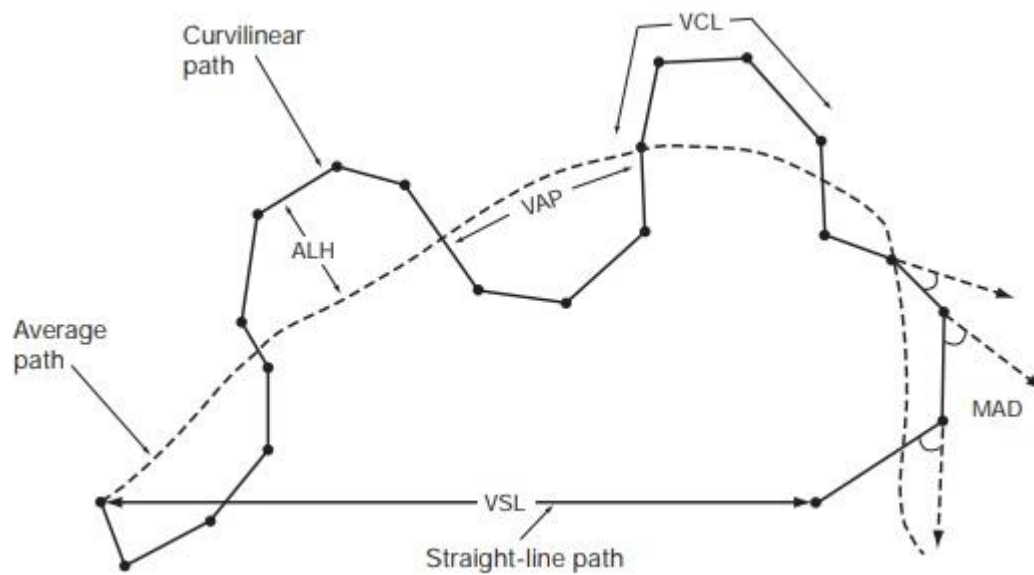
As the result of the semen analysis is leading for the choice of an ART-strategy, good validation of the analysis is crucial. Inadequate validation can result in possible misdiagnosis of the cause of infertility. Subsequently, this can lead to suboptimal results for ongoing treatments, in the form of lower pregnancy rates. Therefore part of this project will focus on validating the current method used by the “Voortplantingsgeneeskunde”-department of the University Medical Center Groningen (UMCG).

For the current UMCG semen analysis, VCM (Volume x Concentration x Motility) is determined by assessing motility of at least 200 spermatozoa and calculating the concentration using a Neubauer counting chamber. This task is, however, too time consuming to use during an IVF or ICSI procedure. Currently, a computer-assisted semen analysis (CASA) is used for that purpose, however this system has shown to not always be accurate in cases of high concentration and distinguishing debris from semen cells. Besides, the CASA is a computer system prone to technical malfunctioning. The current alternative is a rough VCM estimation based on one field of view using a 20X objective. Because it is based on one observation, the error margin of this method will be very large. This project will focus on validating a new, more trustworthy, analogue counting method. Therefore it will be determined how many observations are necessary to get a reliable estimation of the sample's concentration.

#### Computer-assisted semen analysis

The aforementioned CASA system is currently still a widely used tool for semen analysis. Over the years these systems have highly developed, and nowadays most fertility-related laboratories own such a system to help them do their analyses<sup>30</sup>. Not all aspects of the semen analysis can be replaced by a CASA system, but it is very useful for a faster assessment of sperm concentration and motility compared to a manual analysis<sup>31</sup>. Even though it is still highly debated whether CASA can serve as a proper replacement of the manual analysis<sup>32</sup>, analysis of sperm via a CASA system can possibly also be interesting for other purposes. To determine the sperm motility, CASA systems measure a variety of separate motility parameters<sup>33</sup>. Despite the wide variety in systems that are available, the parameters they use are more or less the same. Parameters measured are the curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of average head displacement (ALH) and beat cross frequency (BCF)<sup>34</sup> (see figure 1). Combining this recorded data of patients with their fertilization and pregnancy outcome provides an interesting opportunity to discover a possible correlation between one or more of these parameters and having a successful fertilization.

Previous investigations have suggested a correlation between the VCL, VSL and VAP and the fertilization rate<sup>35,36</sup>. This was assessed, however, for IUI treatments and could therefore be not directly translatable to IVF treatments. Hirano et al., who did research on this parameters in IVF treatments found a correlation between the VCL, VSL and ALH and the subsequent fertilization of harvested oocytes<sup>37</sup>. Most available literature about correlations between these parameters and fertilization, however, has been published almost two decades ago. This, in combination with the limited number of papers addressing this subject, emphasizes the need for more and more recent research. Discovering a significant correlation between one or more of the CASA parameters and fertilization could provide promising means to optimize the fertilization rate of IVF treatments in general and help predicting the chance of a successful treatment. As the UMCG has a database of roughly 10 years of acquired CASA data, this project will also investigate the possible relation between recorded semen characteristics and established fertilization.



*Figure 1 . Variables measured by computer-assisted sperm analysis (CASA) systems. When analyzing a semen sample, a CASA system will measure curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), wobble (WOB = VAP/VCL) and mean angular displacement (MAD). Source: WHO laboratory manual for the Examination and processing of human semen, Fifth edition, fig. 3.3.*

## Material & methods

### Validation Neubauer

In the UMCG semen analysis, concentration is determined by using disposable Neubauer counting chambers. The Neubauer has two separate counting chambers that exist of 9 large grids, all further divided in smaller grids. A concentration assessment is performed in duplo and will start by counting at least one large grid, and more when necessary until at least 200 spermatozoa have been observed. However, which exact grids are counted alters per lab technician. During analyzing of Neubauer samples, suspicion rose that density of cells were higher in fields located in the corner of the counting chamber, compared to fields located in the middle of the counting chamber. Therefore, a series of counts was performed on semen samples used for the counting method validation. Per sample, the amount of spermatozoa was determined for two corner-fields and two middle fields.

### Validation new counting method

To validate the new counting method, immobilized spermatozoa were used. For the validation, fresh semen samples were used that were collected by masturbation into a wide mouth container. The samples were quickly viewed under the microscope to make a rough estimation of the concentration. Based on its estimated concentration, a dilution was made with  $\text{NaHCO}_3$  to immobilize the sperm cells. An estimated concentration of  $>100 \times 10^6$  spermatozoa per ml was diluted 1:40, a concentration between 100 and  $30 \times 10^6$  spermatozoa per ml was diluted 1:20, between 30 and  $15 \times 10^6$  per ml the sample was diluted 1:10 and an estimated concentration of  $<15 \times 10^6$  per ml was diluted 1:5. 10  $\mu\text{l}$  of the sample was loaded into a green counting chamber (*Leja*) and into a disposable Neubauer counting chamber. The green counting chamber was used to count the number of spermatozoa per field of view, with 10 separate observations per sample at different locations equally distributed over the counting chamber. The distinguished locations were the in- and outside of the counting chamber, with one field of view around the edge being the outside, and the upper and lower half of the counting chamber. The observations were done with a 20X objective and, if possible, a 40X objective. To calculate the estimated sample concentration the mean number of counted spermatozoa was calculated for the cumulative number of counted fields of view. This number of spermatozoa was multiplied with the number of times the sample was diluted and subsequently by a factor representing the expected sample-concentration when one spermatozoa per field of view is observed based on the calculated observed volume (*Appendix 1 for calculation of multiplying factor*). For the 20X magnification, this factor was set on 90.000, and for the 40X magnification on 350.000. For example, the concentration estimation for a 1:10 diluted sample observed with a 20X magnification with 15 spermatozoa counted in one field of view is  $15 \times 10 \times 90000 = 13,5 \times 10^6$  spermatozoa per ml. At least 5 minutes after loading the sample, the Neubauer counting chamber was used to determine the reference sample concentration.

### Analysis CASA-dataset

To investigate a possible correlation between semen parameters measured by the CASA and recorded fertilization rates and subsequent pregnancies, a dataset of collected CASA data of patients treated between 2012 and 2017 was analyzed. Analysis was done using R studio. The relevant data of the dataset was selected, being 'Conc\_voor', 'VCM\_voor', 'VCM\_voor\_range', 'VCL\_voor', 'VCL\_na', 'VSL\_voor', 'VSL\_na', 'VAP\_voor', 'VAP\_na', 'ALH\_voor', 'ALH\_na', 'BCF\_voor', 'BCF\_na', 'WOB\_voor', 'WOB\_na', 'NrOva', '2PN', 'OPN', 'NrEmb', 'Zwanger.Binair' and 'Leeftijd t.t.v. behandeling'. A new variable called 'perc.Bevr' was created by dividing the number of '2PN' by the number of 'NrOva', representing the percentage of harvested egg cells that were fertilized. Patients with only 2 or less harvested egg cells were excluded from the dataset as those egg cells are most often of a lowered



quality leading to a natural lower rate of fertilization and a possible risk of bias in the dataset. Furthermore, all patients containing an “N.A.” somewhere in their data were excluded from the dataset as they cannot be used for analysis. A possible correlation with the percentage fertilized egg cells was assessed for all variables except ‘Zwanger.Binair’, ‘2PN’, ‘OPN’ and ‘NrEmb’ because they directly influenced or were directly influenced by the percentage of fertilization. The correlation with a successful pregnancy was assessed for all variables except ‘2PN’, ‘OPN’ and ‘NrEmb’ as they directly influenced or were irrelevant for the achievement of a pregnancy.

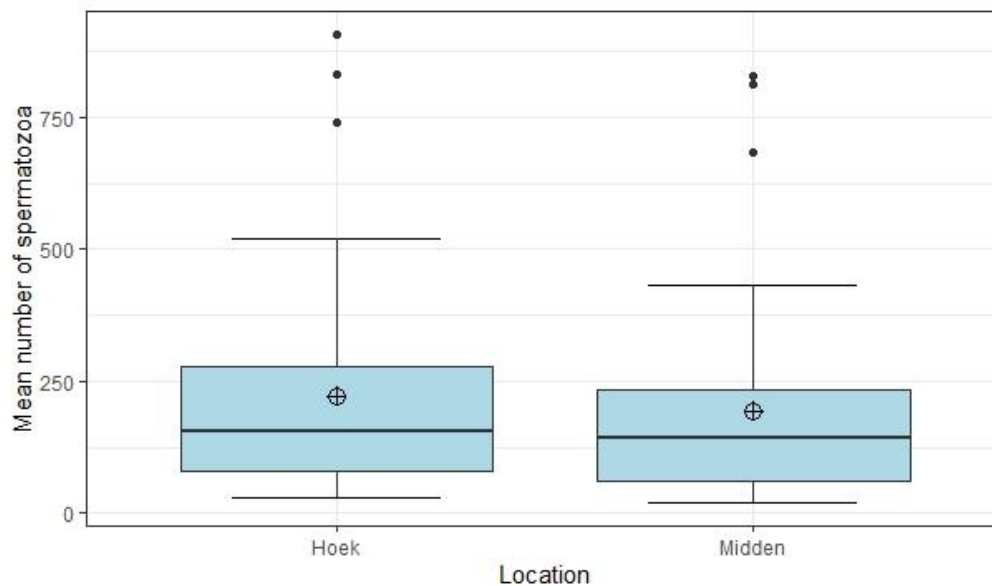
### Statistics

All statistics were performed in R studio (*R studio, Inc.*, version 1.2.5033). Normal distribution of the data was tested using a normality test. The Mann-Whitney U test was used to compare the differences between number of observed spermatozoa in the Neubauer validation, and to test for significant differences between deviances from the Neubauer mean from observations at different locations of a counting chamber. The correlation between semen parameters and oocyte fertilization in the CASA dataset was tested by general linear modelling. Logistic regression was used to test the correlation of semen parameters and successful pregnancies. (*All used R-scripts can be found in Appendix 2 to 6.*)

## Results

### Validation Neubauer

Observations during the semen analysis lead to the hypothesis that counts performed in corner-fields of a Neubauer counting chamber resulted in a higher number of spermatozoa compared to counts performed in middle-fields in the same sample. To test if this observed difference is significant, the mean observed spermatozoa per field was calculated for two observed fields per location per sample (fig. 2). A normality-test revealed that the data is not normally distributed. Therefore a Mann-Whitney U test was performed to test for significance. With a p-value of 0.30, the observed difference in counted spermatozoa appears not to be significant.



*Figure 2 . Comparison of mean number of spermatozoa counted in corner or middle grids of a Neubauer counting chamber. The mean counted numbers were slightly higher for the corner grids, though no significant difference was found (n=78).*

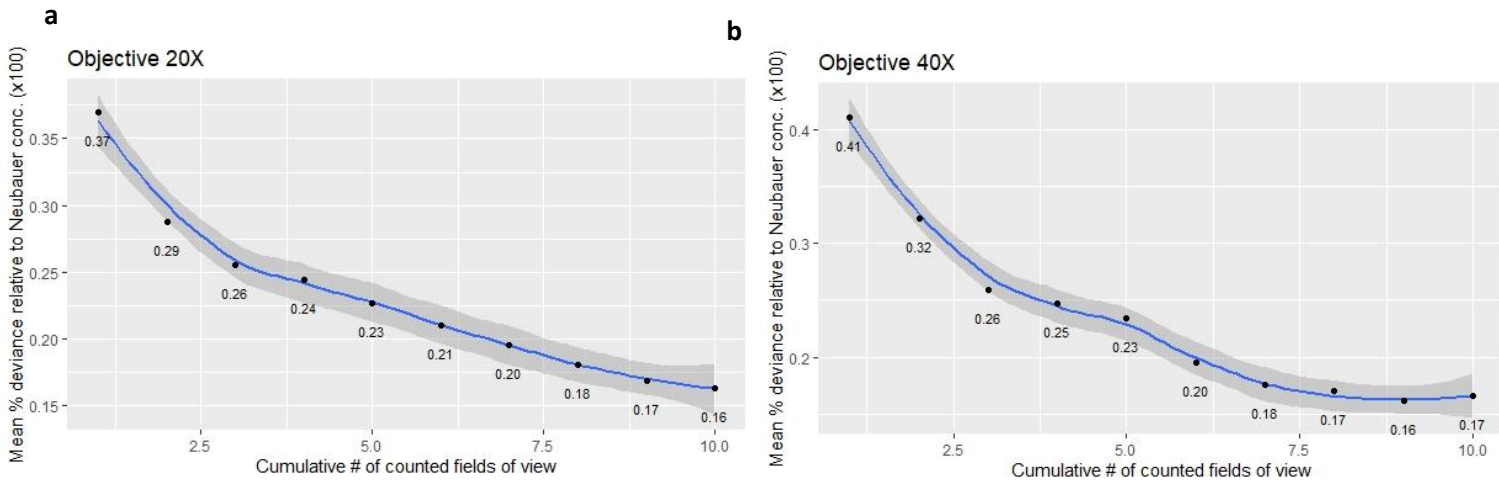
### Validation new counting method

To determine how many separate counts are required to get an acceptable reliable estimation of a sample's concentration, analysis was performed on several fresh semen samples. Per sample the analysis existed of ten separate observations, while alternating between location and magnification.

#### *Number of necessary counts*

To determine the minimum counts that are required for a reliable concentration estimation, the exact number of spermatozoa per field of view was determined for 10 separate fields of view per sample. The cumulative mean of counted sperm cells was determined for 1 to 10 fields of view and this number was used to calculate the estimated concentration. The mean percentage of difference compared to the calculated Neubauer concentration was determined for every cumulative number of counts (fig. 3). When using a 20X objective (A), counting only one field of view will give an estimated concentration that deviates an average 37% of the actual Neubauer concentration. Based on the collective data, a <25% deviation margin to the Neubauer concentration seems like an acceptable norm when also considering time-investment. When we take this acceptable deviation into account, it appears that after counting four different fields of view the deviation of the Neubauer concentration is within acceptable ranges. On the other hand, when using a 40X objective (B) looking at a single field of view

will give a mean difference of 41% when compared to the Neubauer concentration. When handling the acceptable norm of <25% difference to the Neubauer concentration, the norm is met after counting five separate fields of view.

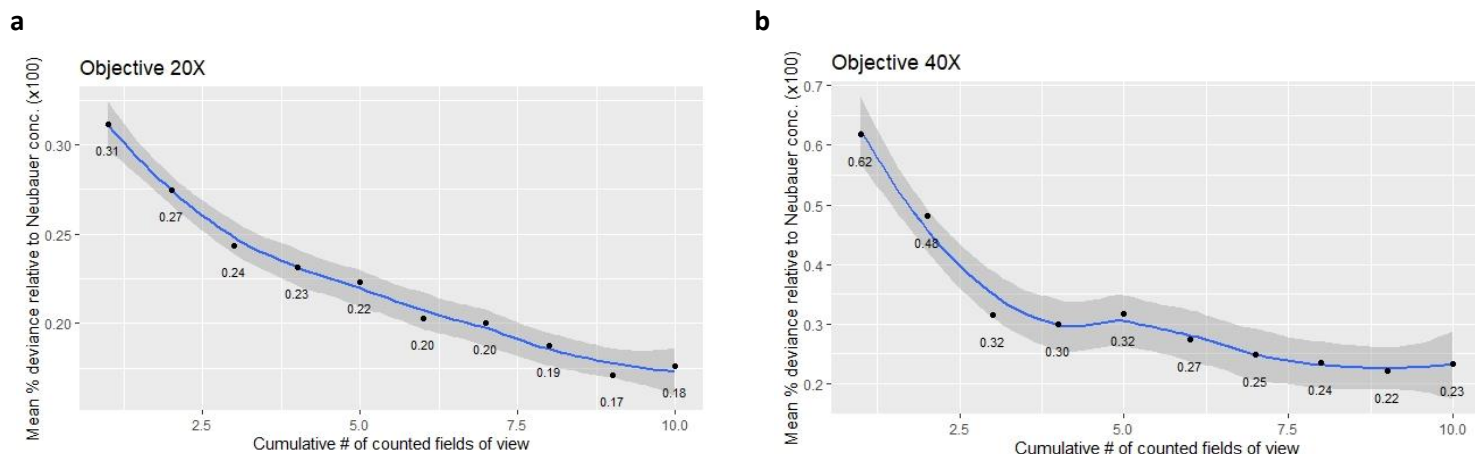


**Figure 3 .** Mean percentage of deviance when compared with the calculated Neubauer concentration per cumulative counted number of fields of view. A) Mean percentage of deviance when using a 20X objective (n=66). The deviance from the Neubauer concentration is 37% when only counting the spermatozoa of one field of view. The acceptable <25% deviance threshold is met after counting four fields of view. B) Mean percentage of deviance when using a 40X objective (n=42). The deviance from the Neubauer concentration is 41% when only counting one field of view. The acceptable <25% deviance threshold is met after counting five fields of view.

#### *Number of necessary counts: Concentration group specific*

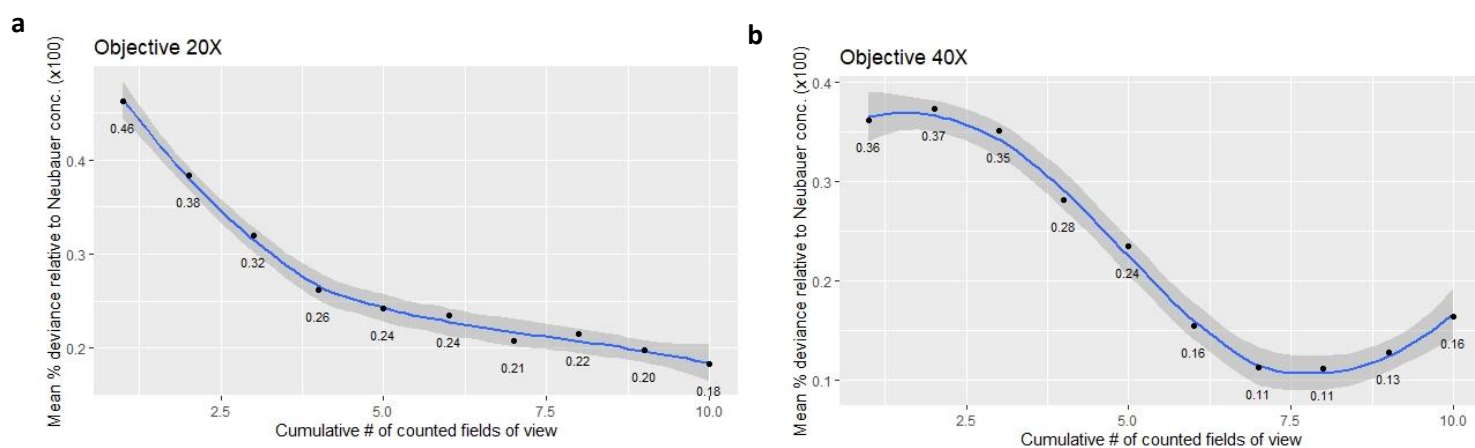
Previous data includes all samples, without distinguishing between different concentration groups. However, spermatozoa-concentration is very variable amongst men. Observing different spermatozoa densities with different objectives might influence the number of counted fields of view needed for a reliable concentration estimation. Therefore the analysis was repeated while distinguishing between concentrations of  $<15 \times 10^6$ ,  $15-30 \times 10^6$  and  $>30 \times 10^6$  spermatozoa per ml.

An explicit difference in accuracy is seen when comparing observing  $<15 \times 10^6$  samples with either a 20X or a 40X objective (fig. 4). When using a 40X objective (fig. 4b), counting a single field of view results in a mean difference of 62% compared to the Neubauer concentration. Furthermore, the threshold of an acceptable <25% difference is only met after eight separate counts. At the same time, using a 20X objective for these samples (fig. 4a) gives a mean difference of 31% compared to the Neubauer concentration. Moreover, the <25% difference threshold is met after three individual counts.



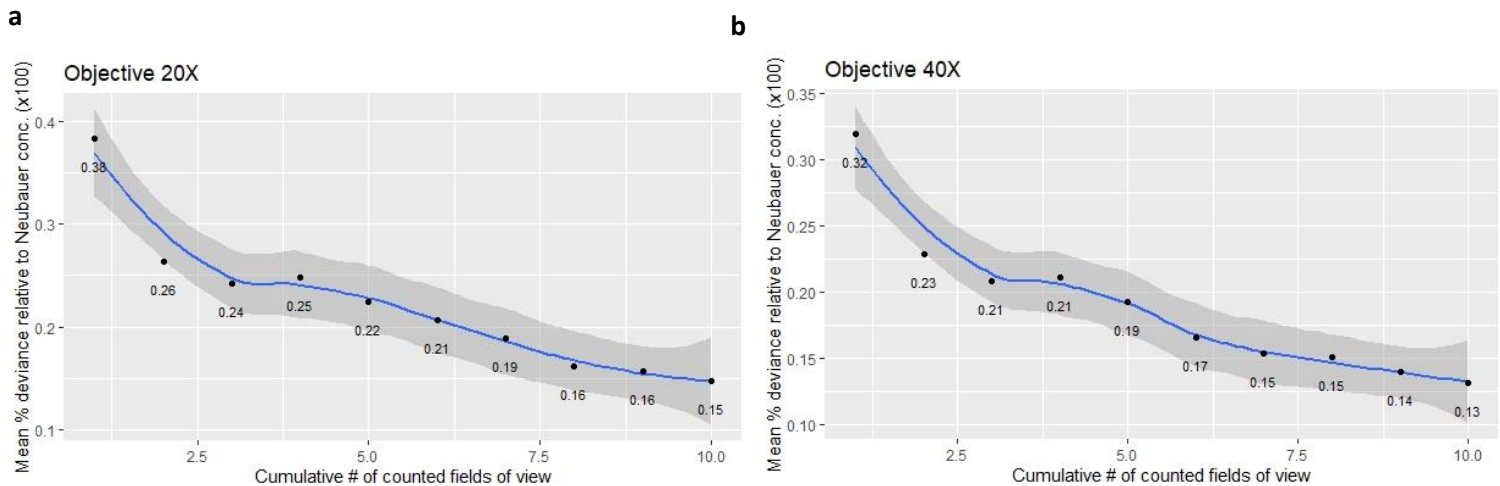
**Figure 4 .** Mean percentage of deviance when compared with the calculated Neubauer concentration per cumulative counted number of fields of view for samples with a concentration of  $<15 \times 10^6$  spermatozoa per ml. A) Mean percentage of deviance when using a 20X objective (n=24). The deviance from the Neubauer concentration is 31% when only counting the spermatozoa of one field of view. The acceptable <25% deviance threshold is met after counting three fields of view. B) Mean percentage of deviance when using a 40X objective (n=12). The deviance from the Neubauer concentration is 62% when only counting one field of view. The <25% deviance threshold is met after counting eight fields of view.

For  $15\text{--}30 \times 10^6$  samples this benefit of using one particular objective is less obvious (fig. 5). Using a 20X objective (fig. 5a) leads to a mean difference of 46% when looking at one field of view, where counting a single field of view with a 40X objective (fig. 5b) results in a 36% mean difference with the Neubauer concentration. For both objectives the <25% difference threshold is met after counting 5 separate fields of view.



**Figure 5 .** Mean percentage of deviance when compared with the calculated Neubauer concentration per cumulative counted number of fields of view for samples with a concentration between  $15 \times 10^6$  and  $30 \times 10^6$  spermatozoa per ml. A) Mean percentage of deviance when using a 20X objective (n=11). The deviance from the Neubauer concentration is 46% when only counting the spermatozoa of one field of view. The acceptable <25% deviance threshold is met after counting five fields of view. B) Mean percentage of deviance when using a 40X objective (n=6). The deviance from the Neubauer concentration is 36% when only counting one field of view. The <25% deviance threshold is met after counting five fields of view.

Lastly, samples with a sperm-concentration of  $>30 \times 10^6$  appear to give the highest accuracy while estimating their concentration (fig. 6). Analyzing the sample using a 20X objective (fig. 6a) results in a difference of 38% compared to the Neubauer concentration when the concentration estimation is based on a single field of view. For a 40X objective (fig. 6b) this is 32%. While the  $<25\%$  difference threshold is met after three separate counts when using the 20X objective, it takes only two separate counts using the 40X objective.

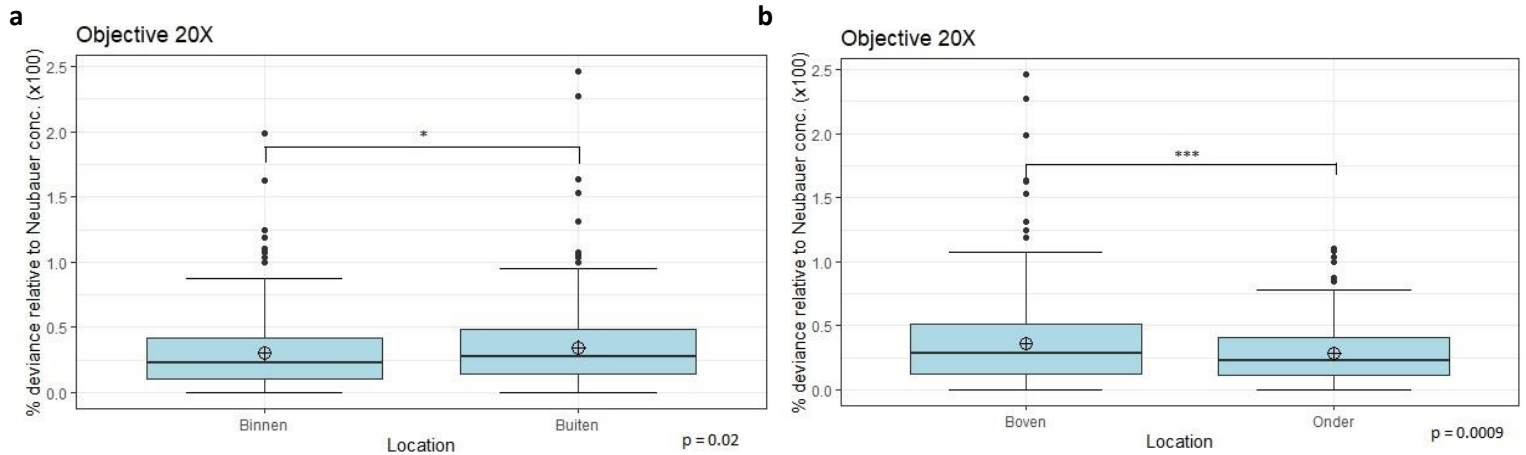


**Figure 6.** Mean percentage of deviance when compared with the calculated Neubauer concentration per cumulative counted number of fields of view for samples with a concentration of  $>30 \times 10^6$  spermatozoa per ml. A) Mean percentage of deviance when using a 20X objective (n=31). The deviance from the Neubauer concentration is 38% when only counting the spermatozoa of one field of view. The acceptable  $<25\%$  deviance threshold is met after counting three fields of view. B) Mean percentage of deviance when using a 40X objective (n=24). The deviance from the Neubauer concentration is 32% when only counting one field of view. The  $<25\%$  deviance threshold is met after counting two fields of view.

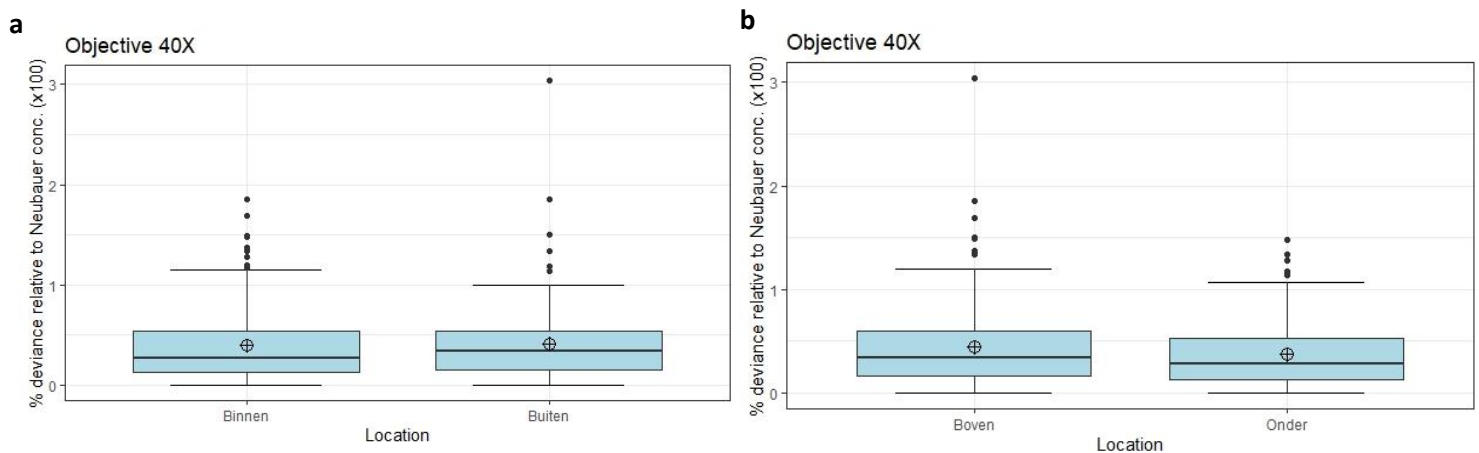
#### Validation of locations

During the semen analysis, the location was recorded for every sample. This data was used to determine possible differences in calculated concentrations from observations in specific locations of the Green counting chamber. A normality test revealed that the data was not normally distributed. Therefore a Mann-Whitney U test was performed to determine significance. First, samples observed with a 20X objective were analyzed (fig. 7). Distinguishing between the in- and outside lead to a mean difference compared to the mean of 30% and 34% respectively. With a p-value of 0.02 it appears that observations made in the inside of the counting chamber have a lower deviance from the mean than those in the outside. In addition, observations done in the upper half resulted in 37% difference from

the Neubauer mean, where those in the lower half had a mean deviance of 27%. The p-value of 0.0009 confirmed that the lower half observations have a significant reduced deviance.



**Figure 7 .** Comparison of mean deviances from observations performed in different locations of the green counting chamber with use of a 20X objective. A) Mean deviances from observations performed in inner or outer area (1 field of view around the edge) of the counting chamber. Observations done in the inner area had a mean deviance of 30%. Observations done in the outer area had a mean deviance of 34%. The deviance appeared to be significantly higher for observations in the outer area of the counting chamber ( $p = 0.02$ ). B) Mean deviances from observations performed in the upper or lower half of the green counting chamber. Observations done in the upper half of the counting chamber had a 37% mean deviance from the Neubauer concentration where those in the lower half of the counting chamber had a mean deviance of 27%. The observations in the lower half had a significant lower deviance than those in the upper half ( $p = 0.0009$ )



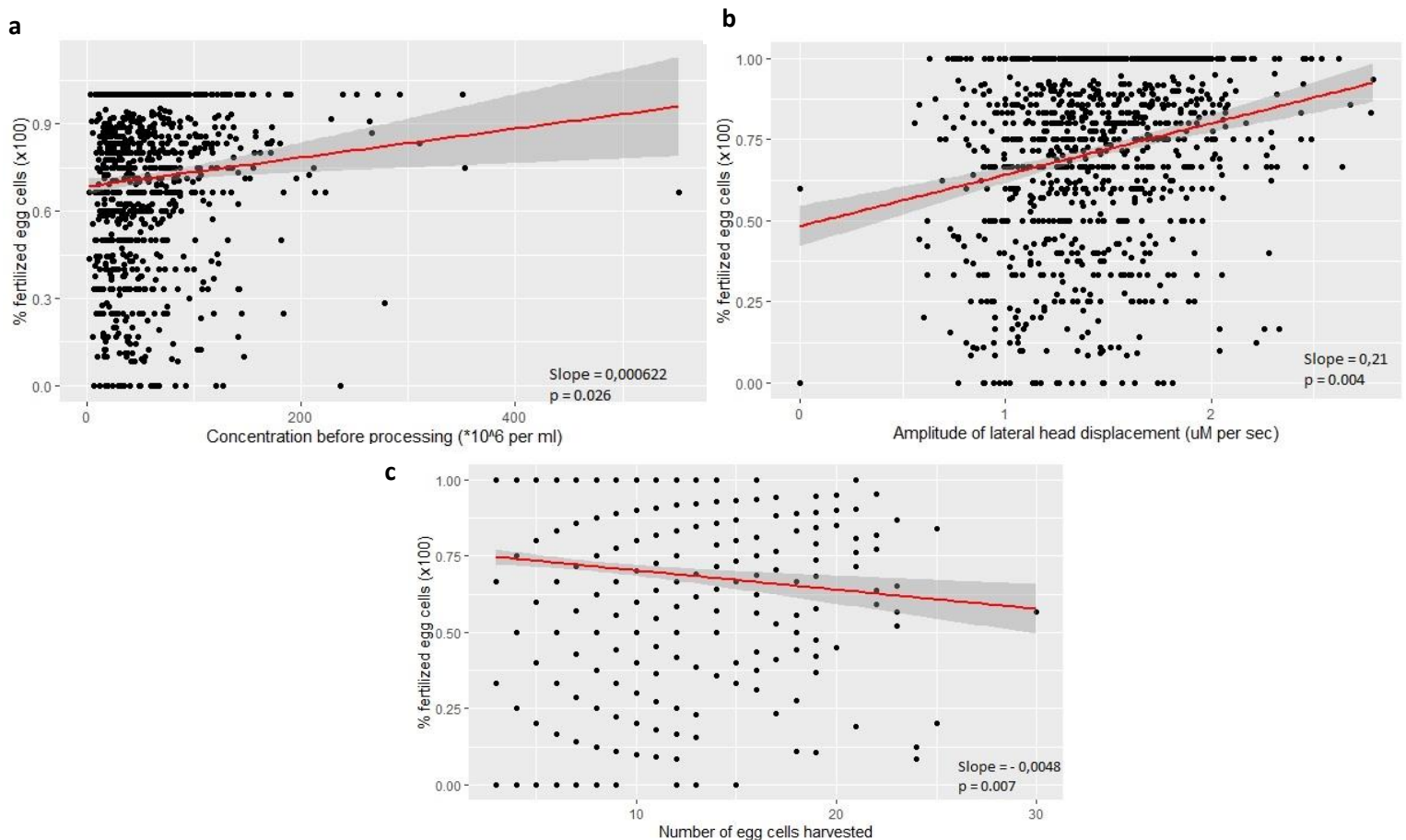
**Figure 8.** Comparison of mean deviances from observations performed in different locations of the green counting chamber with use of a 40X objective. A) Mean deviances from observations performed in inner or outer area (1 field of view around the edge) of the counting chamber. Observations done in the inner area had a mean deviance of 39%. Observations done in the outer area had a mean deviance of 41%. The observed difference was not significant ( $p = 0.19$ ). B) Mean deviances from observations performed in the upper or lower half of the green counting chamber. Observations done in the upper half of the counting chamber had a 44.5% mean deviance from the Neubauer concentration where those in the lower half of the counting chamber had a mean deviance of 37%. Although a trend towards lower deviance in the lower half was observed, the difference was not significant ( $p = 0.07$ ).



Subsequently, the same analysis was performed for observations where the 40X objective was used (fig. 8). When using a 40X objective, observations done at the inside of the counting chamber lead to a mean 39% deviance from the Neubauer concentration. Observations at the outside of the chamber had a mean deviance of 41%. A p-value of 0.19 revealed that this difference was not significant. Again, differences between the upper and lower half of the counting chamber were observed. Observation in the upper half gave a mean deviance of 44,5% where lower half observation had a mean difference of 37%. A p-value of 0.07 showed that although there seems to be a trend of lower deviance within lower half observations, this difference is not significant.

### Analysis CASA-dataset

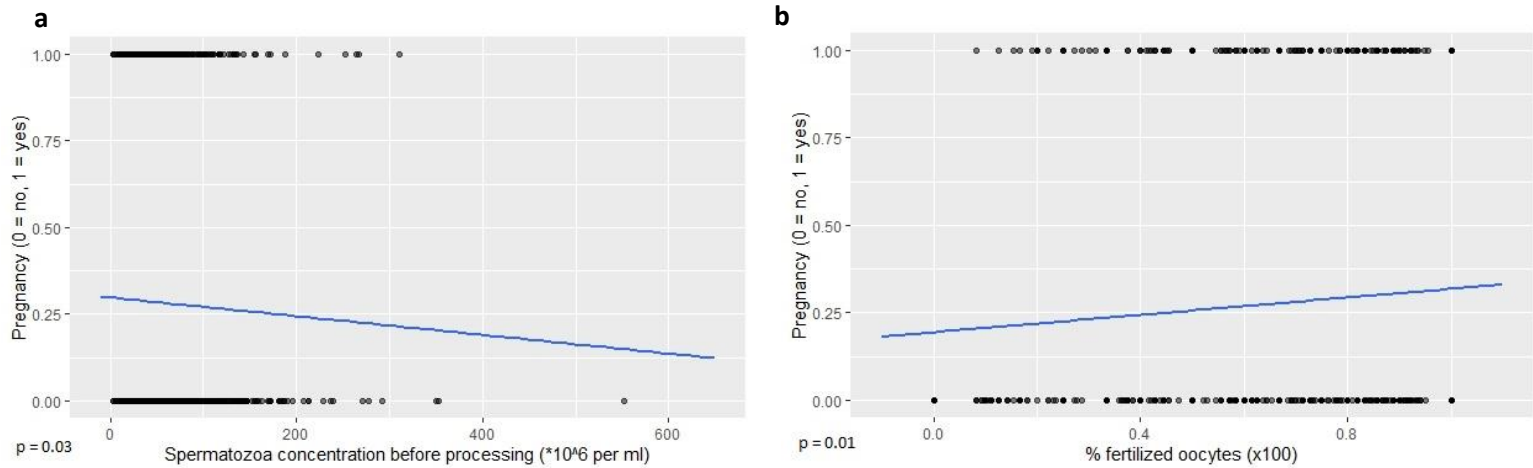
To investigate the possible correlation between semen parameters and fertilization, collected CASA data of patients treated between 2012 and 2017 was analyzed. The correlation between the parameters and percentage of fertilized egg cells was evaluated using general linear modelling (fig. 9). A significant effect was found for the spermatozoa concentration before processing (fig. 9a), the ALH after processing (fig. 9b) and the total number of oocytes that were harvested (fig. 9c) with a p-value of respectively 0.026, 0.004 and 0.007. Although not significant, a trend towards an effect was seen for the VSL and VAP before processing (data not shown). Regarding the concentration, a higher concentration of spermatozoa before processing the sample correlates with a higher percentage of fertilized oocytes. Similarly, a higher ALH leads to a higher percentage of fertilized oocytes. On the



**Figure 9.** General linear models testing correlation between semen parameters and fertilization rate. A significant correlation between variables and percentage of fertilized oocytes was found for spermatozoa concentration before processing the sample (A,  $p = 0.026$ ), ALH after processing (B,  $p = 0.004$ ) and the total number of oocytes that were harvested (C,  $p = 0.007$ ).

other hand, according to the calculation in *R*, the more oocytes are harvested the smaller the percentage of fertilized oocytes.

The possible correlation between any parameters and a successful pregnancy was then evaluated using logistic regression in *R* (fig. 10). A possible effect was found for the spermatozoa concentration before processing of the sample (fig. 10a) and for the total percentage of fertilized oocytes (fig. 10b) with a p-value of 0.03 and 0.01 respectively. A non-significant trend was seen for VCM before sample processing with a p-value of 0.096 (data not shown).



*Figure 10* . Logistic regression models testing correlation between semen parameters and successful pregnancies. A significant correlation between variables and a successful pregnancy was found for spermatozoa concentration before processing the sample (A,  $p = 0.03$ ) and the percentage of fertilized oocytes (B,  $p = 0.01$ ).

## Discussion

Infertility is a big worldwide health problem. Assisted reproduction techniques (ART), such as IUI, IVF and ICSI, made it possible for couples suffering from infertility to become pregnant. Prior to starting an ART treatment, men have to undergo a semen analysis to assess the quality of their semen. This analysis exists of different macro- and microscopic observations of a fresh semen sample, including concentration and motility. As the semen analysis is leading in choosing an ART strategy, naturally it is important that this analysis is correct and well validated. This project therefore aimed to validate the current semen analysis used by the “Voortplantingsgeneeskunde” department of the UMCG.

### Validation of the Neubauer counting chamber

The Neubauer counting chamber is normally used to determine the spermatozoa concentration of a sample. After observations, suspicion rose that in the same Neubauer counting chamber, counts performed in corner grids lead to a higher number of counted spermatozoa than those performed in middle grids. The observed difference in counted spermatozoa appeared not to be significant. Even though the middle grid is the most popular location to start counting amongst lab technicians, counts performed in corner grids will not affect the concentration determination significantly.

### Validation new counting method

#### *Number of counts and objectives*

To validate the new counting method it had to be determined how many fields of view need to be counted to give a reliable estimation of the sample's concentration. When using <25% deviance with the Neubauer mean concentration as the acceptable threshold, you would have to count the spermatozoa in four separate fields of view when using a 20X objective and five separated counts are required when using a 40X objective. In this case, it would be recommended to only use the 20X objective. This is, however, only true when considering the dataset as a whole.

As the sperm-concentrations are highly variable amongst men, more accurate results can be achieved when looking at the most optimal conditions per concentration group. Starting with samples with a concentration of  $<15 \times 10^6$  spermatozoa per ml, there is a substantial difference seen in accuracy when using a 20X objective compared to the 40X objective. Using the 40X objective in this concentration group requires eight separate counts before the <25% deviance threshold is reached, opposing only three counts using the 20X objective to reach the same accuracy. Therefore it is advised to take the mean observed spermatozoa of three separate fields of view, observed with a 20X objective to reach the highest accuracy in the shortest time-span.

Continuing to concentrations ranging from 15 to  $30 \times 10^6$  spermatozoa per ml, the difference is less obvious. For both objectives the <25% deviance threshold is met after counting of 5 separate fields of view. Considering convenience, using a 40X objective is preferred, as this requires the counting of less sperm cells per field of view which eventually leads to a smaller time investment.

Lastly, samples with a concentration of  $>30 \times 10^6$  spermatozoa per ml seem to cause the least effort to achieve accuracy. The <25% deviance threshold is met after three separate counts when using the 20X objective, and even after two separate counts the threshold is met using the 40X objective. Thus, for samples that are estimated at  $>30 \times 10^6$  spermatozoa per ml, using the 40X objective leads to an acceptable accuracy in two separate counts.

It needs to be noted that the used <25% deviance threshold is still a relatively big difference with the actual concentration as observed with the Neubauer counting chamber. This number was handled however with the eye on highest accuracy within the shortest amount of time. Nevertheless, it could be beneficial to further explore how to lower the overall deviance with the Neubauer concentration.

Perhaps, further optimizing the factor used to determine how many spermatozoa are present in one ml after observing one spermatozoa in a field of view could provide a solution for narrowing down the mean deviance.

#### Location

As the location of individual counts was documented, it was also analyzed if location of an observation influenced the accuracy of a concentration estimation. Per objective (20X or 40X) the influence of observations in the in- or outside and upper or lower half of the counting chamber on the calculated concentration was assessed.

Starting with the 20X objective, counts performed on the inside had a slightly lower deviance compared to those in the outside border of the counting chamber. When comparing the upper and lower half, it is obvious that observations done in the lower half have a significant lower deviance than those in the upper half of the counting chamber. Likely, observations done within the outer border and the lower half of the counting chamber have the highest accuracy.

Remarkably, this is different when using the 40X objective. Despite a trend towards lower deviance of observations in the lower half of a counting chamber, none of the differences between locations is significant. This is remarkable as the only difference between a lot of observations is just the used objective, not the sample itself. Possibly, because looking at a smaller volume automatically leads to the observation of less spermatozoa at the same time, the difference in observed sperm cells is also naturally smaller.

The current recommendations are done assuming the samples are analyzed using the whole field of view. Nevertheless, it might be even less time consuming when the number of spermatozoa is counted using only the grid present on the ocular. It will take a shorter time to count all present cells in the grid, as the surface of the grid is smaller than the whole field of view. It is therefore recommended that a similar validation will be executed for when spermatozoa are counted using the ocular's grid. Though, it needs to be monitored whether using the whole field of view is not more time-efficient and more accurate for the different concentration groups. Furthermore, the final goal of this validation is to not only accurately estimate the concentration, but the total VCM. Therefore, future research should include motile cells to this validation method. Instead of diluted samples, fresh undiluted semen samples should be used, analyzing how many fields of view and which objectives are required to not only accurately estimate the concentration but also motility of the sperm cells.

#### Analysis CASA-dataset

A CASA-dataset containing the data of 5 years of patients was analyzed to discover a possible correlation between semen parameters and fertilization. General linear modelling revealed that the higher the spermatozoa concentration before processing of the sample was, the higher the percentage of fertilized egg cells. The same goes for ALH after processing, the higher the ALH, the higher the percentage of fertilized egg cells. An increase of ALH is associated with hyperactivation of sperm cells<sup>38</sup>. This might suggest that sperm cells of those patients had a higher rate of hyperactivation. As hyperactivation is necessary to successfully fertilize an oocyte, it is likely that semen containing more cells that successfully achieve hyperactivation has a higher chance of actually fertilizing the oocyte. Interestingly, the find of a correlation between ALH and fertilized oocytes corresponds with the correlation that was found by Hirano and his colleagues in 2001.<sup>37</sup> A significant correlation was also found for the number of harvested oocytes. More oocytes would lead to a lower percentage of fertilized oocytes. However, when visually analyzing the graph of this data, it looks like more harvested

oocytes lead to a higher fertilization rate. Perhaps, the non-normal distributed nature of the data affects the accuracy of the linear model. An interestingly absent factor is the age of the woman during the treatment. It is repeatedly found in literature that an increased age of the woman (>35) affects the chance to conceive negatively as less oocytes are available and the remaining ones have higher chance of being of bad quality<sup>39</sup>. Though, the exact mechanism of decreased fecundity by advanced maternal age is unknown. Perhaps, factors that cause age-related decreased fecundity do not interfere with the initial fertilization of an oocytes by a sperm cell.

Then, when investigating correlations between the parameters and a successful pregnancy, a significant effect was found for spermatozoa concentration before sample processing and the total percentage of fertilized oocytes after insemination. However, these results appear to be fairly debatable. The correlations were evaluated using logistic regression, normally generating a sigmoidal curve. Nevertheless, after plentiful attempts using *R*, a sigmoidal curve was not successfully reproduced for this specific dataset. Overall, the data in this form does not seem to fit the linear or the logistic regression models very perfectly. Ideally, the data is optimized to fit a certain model by only selecting the variables that provide the strongest model possible, and eliminate the variables that are irrelevant to its strength. As identifying a possible correlation between semen parameters and fertilization might be of great use to predict the chance of a successful treatment, it is recommended that future research further investigates what are the best fitted models to discover this correlation. A general remark about the CASA data-set is that the norm for proceeding with an IVF treatment instead of ICSI has changed over the years within the UMCG. Where the threshold for IVF first was a VCM of at least  $60 \cdot 10^6$ , this was adjusted to a VCM of at least  $30 \cdot 10^6$ . Nowadays it is even shifting towards a VCM of  $15 \cdot 10^6$ . Because of the norm being  $60 \cdot 10^6$  for a long period of time during the collection of the currently used data, the data might be somewhat biased. To overcome this possible bias it might be recommended to select data from a wider timespan, including more recent data. Furthermore, collecting more data over the upcoming years and including this in the current dataset might be favorable in preventing a possible bias.

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## Appendix 1: Calculation of multiplying factor semen concentration

Field of view of a 20x objective (10/22) is 0,11 cm ( $10 \times 20 = 200$ ,  $22/200$ ).

Surface =  $\pi r^2 \rightarrow 3.14 \times 0,055^2 = 0,0095$

Depth of counting chamber =  $12 \mu\text{m} = 0,0012 \text{ cm}$ .

Volume in 1 field of view =  $0,0095 \times 0,0012 = 0,00000113982 \text{ cm}^3 = 11.3 \text{ nl}$ .

Ergo, 1 spermatozoon in a 20x field of view equals 87.733/ml  $\rightarrow$  round up to 90000/ml

Field of view of 40x objective is 4 times as small as 20x objective  $\rightarrow$  1 spermatozoon in FoV is 350.934 per ml  $\rightarrow$  round up to 350000/ml

## Appendix 2: R-script for detection of differences in Neubauer counting chamber

```
data.neub <- read.csv2("SA_Neubauer.csv", header = TRUE)

#####

# Normaliteit controleren

#####

library("gmodels")
library("car")
library("DescTools")
library("ggplot2")
library("qqplotr")
library("dplyr")

# str(data.neub$Locatie) = factor, dat klopt
#Descriptive statistics maken van de dataset
data.neub %>% select(Locatie, gem.aant.cellen) %>% group_by(Locatie) %>%
  summarise(n = n(),
            mean = mean(gem.aant.cellen, na.rm = TRUE),
            sd = sd(gem.aant.cellen, na.rm = TRUE),
            stderr = sd/sqrt(n),
            LCL = mean - qt(1 - (0.05 / 2), n - 1) * stderr,
            UCL = mean + qt(1 - (0.05 / 2), n - 1) * stderr,
            median = median(gem.aant.cellen, na.rm = TRUE),
            min = min(gem.aant.cellen, na.rm = TRUE),
            max = max(gem.aant.cellen, na.rm = TRUE),
            IQR = IQR(gem.aant.cellen, na.rm = TRUE),
            LCLmed = MedianCI(gem.aant.cellen, na.rm=TRUE)[2],
            UCLmed = MedianCI(gem.aant.cellen, na.rm=TRUE)[3])

# Boxplot maken om te kijken naar eventuele outliers
ggplot(data.neub, aes(x = Locatie, y = gem.aant.cellen, fill = Locatie)) +
  stat_boxplot(geom = "errorbar", width = 0.5) +
  geom_boxplot(fill = "light blue") +
  stat_summary(fun.y=mean, geom="point", shape=10, size=3.5, color="black") +
```

```

theme_bw() + theme(legend.position="none")+
xlab("Location") +
ylab("Mean number of spermatozoa")

# Dan testen op normaliteit
data.neub %>% group_by(Locatie) %>%
  summarise(`W Stat` = shapiro.test(gem.aant.cellen)$statistic,
            p.value = shapiro.test(gem.aant.cellen)$p.value)

# p-waardes voor de dataset is <0.05
# dus data is niet normaal verdeeld

# Met een qq-plot kun je dit visualiseren
ggplot(data = data.neub, mapping = aes(sample = gem.aant.cellen, color = Locatie, fill = Locatie)) +
  stat_qq_band(alpha=0.5, conf=0.95, qtype=1, bandType = "boot") +
  stat_qq_line(identity=TRUE) +
  stat_qq_point(col="black") +
  facet_wrap(~ Locatie, scales = "free") +
  labs(x = "Theoretical Quantiles", y = "Sample Quantiles") + theme_bw()

# Ook in qqplots dus duidelijk niet normaal verdeeld, datapunten
# zouden de lijn moeten volgen, is niet het geval

# Daarom dus Mann-Whitney-U test uitvoeren (Wilcox-test)
m.neub<-wilcox.test(gem.aant.cellen ~ Locatie, data=data.neub, na.rm=TRUE, paired=FALSE,
exact=FALSE, conf.int=TRUE)

print(m.neub)

#Hodges Lehmann Estimator (hoeveel meer of minder te verwachten)
m.neub$estimate

# Deze MWU-test zegt dat ondanks het verschil in gemiddeld getelde cellen
# in een midden- of hoekveld, deze niet significant is.

```

### Appendix 3: R-script for analysis new counting method: Number of required fields of view (20X objective)

```
#rm(list = ls())

# VOOR 20X VERGROTING!!

library(ggplot2)

# veranderen van output decimalen in R:
options(digits = 3)

#

CI_data20 <- read.csv2("concafwijkperaantvelden20.csv", header = TRUE)

#sample size
n <- length(CI_data20$Samplenr)

#####

# Variabele voor concentraties maken
#####

extractConc <- function(Conc) {
  Conc <- as.numeric(Conc)

  if ((Conc>15000000) & (Conc<30000000)) {
    return("30-15")
  } else if (Conc>=30000000) {
    return(">30")
  } else if (Conc<=15000000) {
    return("<15")
  } else {
    return("Other")
  }
}

concentrations <- NULL
for (i in 1:nrow(CI_data20)) {
  concentrations <- c(concentrations, extractConc(CI_data20[i, "X.Neubauer."]))
}
```



```

}
CI_data20$Conc <- as.factor(concentrations)

#####

# mean en CI uitrekenen

#####

firstCol <- which( colnames(CI_data20)=="X1x" )
meanArray <- NULL
seArray <- NULL
meanArray30 <- NULL
meanArray15 <- NULL
meanArray3015 <- NULL
seArray30 <- NULL
seArray15 <- NULL
seArray3015 <- NULL

for (i in firstCol:(firstCol+9)){
  meanArray <- c(meanArray, mean(abs(CI_data20[,i])))
  seArray <- c(seArray, sd(abs(CI_data20[,i]))/sqrt(n))

  data30 <- CI_data20[CI_data20[, "Conc"] == ">30", i]
  data15 <- CI_data20[CI_data20[, "Conc"] == "<15", i]
  data3015 <- CI_data20[CI_data20[, "Conc"] == "30-15", i]

  meanArray30 <- c(meanArray30, mean(abs(data30)))
  meanArray15 <- c(meanArray15, mean(abs(data15)))
  meanArray3015 <- c(meanArray3015, mean(abs(data3015)))

  seArray30 <- c(seArray30, sd(abs(data30))/sqrt(length(data30)))
  seArray15 <- c(seArray15, sd(abs(data15))/sqrt(length(data15)))
  seArray3015 <- c(seArray3015, sd(abs(data3015))/sqrt(length(data3015)))
}

```

```

conf.level <- 0.9

z <- qt((conf.level+1)/2, df=n-1)
z30 <- qt((conf.level+1)/2, df=length(data30)-1)
z15 <- qt((conf.level+1)/2, df=length(data15)-1)
z3015 <- qt((conf.level+1)/2, df=length(data3015)-1)

CIArray <- seArray * z
CIArray30 <- seArray30 * z30
CIArray15 <- seArray15 * z15
CIArray3015 <- seArray3015 * z3015
summaryStat <- cbind(meanArray, seArray, CIArray, meanArray30,
                      seArray30, CIArray30, meanArray15, seArray15,
                      CIArray15, meanArray3015, seArray3015, CIArray3015)
dataStats20 <- as.data.frame(summaryStat)

#####
# Grafiekje maken
#####

# Basic line plot with points
ggplot(data=dataStats20, aes(x=1:10, y=meanArray, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 20X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)") +
  geom_text(aes(label=sprintf("%0.2f", round(meanArray, digits = 2))), size = 3, hjust = 0.5, vjust = 3,
position = "identity")

ggplot(data=dataStats20, aes(x=1:10, y=meanArray15, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +

```

```

ggtitle("Objective 20X") +
xlab("Cumulative # of counted fields of view") +
ylab("Mean % deviance relative to Neubauer conc. (x100)")+
  geom_text(aes(label=sprintf("%.2f", round(meanArray15, digits = 2))), size = 3, hjust = 0.5, vjust =
3, position = "identity")

```

```

ggplot(data=dataStats20, aes(x=1:10, y=meanArray3015, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 20X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)")+
  geom_text(aes(label=sprintf("%.2f", round(meanArray3015, digits = 2))), size = 3, hjust = 0.5, vjust =
= 3, position = "identity")

```

```

ggplot(data=dataStats20, aes(x=1:10, y=meanArray30, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 20X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)")+
  geom_text(aes(label=sprintf("%.2f", round(meanArray30, digits = 2))), size = 3, hjust = 0.5, vjust =
3, position = "identity")

```

#### Appendix 4: R-script for analysis new counting method: Number of required fields of view (40X objective)

# VOOR 40X VERGROTING!!

```
library(ggplot2)
```

```
CI_data40 <- read.csv2("concafwijkperaantvelden40.csv", header = TRUE)
```

```
#sample size
```

```
n <- length(CI_data40$Samplenr)
```

```
#####
```

```
# Variabele voor concentraties maken
```

```
#####
```

```
extractConc <- function(Conc) {
```

```
  Conc <- as.numeric(Conc)
```

```
  if ((Conc>15000000) & (Conc<30000000)) {
```

```
    return("30-15")
```

```
  } else if (Conc>=30000000) {
```

```
    return(">30")
```

```
  } else if (Conc<=15000000) {
```

```
    return("<15")
```

```
  } else {
```

```
    return("Other")
```

```
  }
```

```
}
```

```
concentrations <- NULL
```

```
for (i in 1:nrow(CI_data40)) {
```

```
  concentrations <- c(concentrations, extractConc(CI_data40[i, "X.Neubauer."]))
```

```
}
```

```
CI_data40$Conc <- as.factor(concentrations)
```

```
#####

# mean en CI uitrekenen

#####

firstCol <- which( colnames(CI_data40)=="X1x" )
meanArray <- NULL
seArray <- NULL
meanArray30 <- NULL
meanArray15 <- NULL
meanArray3015 <- NULL
seArray30 <- NULL
seArray15 <- NULL
seArray3015 <- NULL

for (i in firstCol:(firstCol+9)){
  meanArray <- c(meanArray, mean(abs(CI_data40[,i])))
  seArray <- c(seArray, sd(abs(CI_data40[,i]))/sqrt(n))

  data30 <- CI_data40[CI_data40[, "Conc"] == ">30", i]
  data15 <- CI_data40[CI_data40[, "Conc"] == "<15", i]
  data3015 <- CI_data40[CI_data40[, "Conc"] == "30-15", i]

  meanArray30 <- c(meanArray30, mean(abs(data30)))
  meanArray15 <- c(meanArray15, mean(abs(data15)))
  meanArray3015 <- c(meanArray3015, mean(abs(data3015)))

  seArray30 <- c(seArray30, sd(abs(data30))/sqrt(length(data30)))
  seArray15 <- c(seArray15, sd(abs(data15))/sqrt(length(data15)))
  seArray3015 <- c(seArray3015, sd(abs(data3015))/sqrt(length(data3015)))
}

conf.level <- 0.9

z <- qt((conf.level+1)/2, df=n-1)
```

```

z30 <- qt((conf.level+1)/2, df=length(data30)-1)
z15 <- qt((conf.level+1)/2, df=length(data15)-1)
z3015 <- qt((conf.level+1)/2, df=length(data3015)-1)

CIArray <- seArray * z
CIArray30 <- seArray30 * z30
CIArray15 <- seArray15 * z15
CIArray3015 <- seArray3015 * z3015

summaryStat <- cbind(meanArray, seArray, CIArray, meanArray30,
                     seArray30, CIArray30, meanArray15, seArray15,
                     CIArray15, meanArray3015, seArray3015, CIArray3015)

dataStats <- as.data.frame(summaryStat)

#####
# Grafiekje maken
#####

# Basic line plot with points
ggplot(data=dataStats, aes(x=1:10, y=meanArray, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 40X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)") +
  geom_text(aes(label=sprintf("%0.2f", round(meanArray, digits = 2))), size = 3, hjust = 0.5, vjust = 3,
            position = "identity")

ggplot(data=dataStats, aes(x=1:10, y=meanArray15, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +

```



```

ggtitle("Objective 40X") +
xlab("Cumulative # of counted fields of view") +
ylab("Mean % deviance relative to Neubauer conc. (x100)") +
  geom_text(aes(label=sprintf("%.2f", round(meanArray15, digits = 2))), size = 3, hjust = 0.5, vjust =
3, position = "identity")

```

```

ggplot(data=dataStats, aes(x=1:10, y=meanArray3015, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 40X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)") +
  geom_text(aes(label=sprintf("%.2f", round(meanArray3015, digits = 2))), size = 3, hjust = 0.5, vjust =
= 3, position = "identity")

```

```

ggplot(data=dataStats, aes(x=1:10, y=meanArray30, group=1)) +
  geom_smooth(method = "loess", ) +
  geom_point() +
  ggtitle("Objective 40X") +
  xlab("Cumulative # of counted fields of view") +
  ylab("Mean % deviance relative to Neubauer conc. (x100)") +
  geom_text(aes(label=sprintf("%.2f", round(meanArray30, digits = 2))), size = 3, hjust = 0.5, vjust =
3, position = "identity")

```

## Appendix 5: R-script for analysis new counting method: Differences between locations of the counting chamber

```
data20 <- read.csv2("SA_locaties.csv")

#####

# Kijken of Mann-Whitney-U test nodig is
# voor niet normaal verdeelde data
#####

library("gmodels")
library("car")
library("DescTools")
library("ggplot2")
library("qqplotr")
library("dplyr")

# binbui moet een factor zijn, als het goed is staat deze al zo
# Data zonder NA's genereren
data20noNA <- data20 %>% filter(rowSums(is.na(data20)) == 0)

#Descriptive statistics maken van de dataset
data20noNA %>% select(binbui, perc.Verschil) %>% group_by(binbui) %>%
  summarise(n = n(),
            mean = mean(abs(perc.Verschil), na.rm = TRUE),
            sd = sd(abs(perc.Verschil), na.rm = TRUE),
            stderr = sd/sqrt(n),
            LCL = mean - qt(1 - (0.05 / 2), n - 1) * stderr,
            UCL = mean + qt(1 - (0.05 / 2), n - 1) * stderr,
            median = median(abs(perc.Verschil), na.rm = TRUE),
            min = min(abs(perc.Verschil), na.rm = TRUE),
            max = max(abs(perc.Verschil), na.rm = TRUE),
            IQR = IQR(abs(perc.Verschil), na.rm = TRUE),
            LCLmed = MedianCI(abs(perc.Verschil), na.rm=TRUE)[2],
```

```
UCLmed = MedianCI(abs(perc.Vershil), na.rm=TRUE)[3])
```

```
# Boxplot maken om te kijken naar eventuele outliers
```

```
ggplot(data20noNA, aes(x = binbui, y = abs(perc.Vershil), fill = binbui)) +  
  stat_boxplot(geom = "errorbar", width = 0.5) +  
  geom_boxplot(fill = "light blue") +  
  stat_summary(fun.y=mean, geom="point", shape=10, size=3.5, color="black") +  
  ggtitle("Objective 20X") +  
  theme_bw() + theme(legend.position="none") +  
  xlab("Location") +  
  ylab("% deviance relative to Neubauer conc. (x100)")
```

```
# Dan testen op normaliteit
```

```
data20noNA %>% group_by(binbui) %>%  
  summarise(`W Stat` = shapiro.test(abs(perc.Vershil))$statistic,  
            p.value = shapiro.test(abs(perc.Vershil))$p.value)
```

```
# p-waardes voor zowel de normale als de absolute dataset is <0.05
```

```
# dus data is niet normaal verdeeld
```

```
# Met een qq-plot kun je dit visualiseren
```

```
ggplot(data = data20noNA, mapping = aes(sample = abs(perc.Vershil), color = binbui, fill = binbui)) +  
  stat_qq_band(alpha=0.5, conf=0.95, qtype=1, bandType = "boot") +  
  stat_qq_line(identity=TRUE) +  
  stat_qq_point(col="black") +  
  facet_wrap(~ binbui, scales = "free") +  
  labs(x = "Theoretical Quantiles", y = "Sample Quantiles") + theme_bw()
```

```
# Ook in qqplots dus duidelijk niet normaal verdeeld, datapunten
```

```
# zouden de lijn moeten volgen, is niet het geval
```

```
# Daarom dus Mann-Whitney-U test uitvoeren (Wilcox-test)
```

```

m1<-wilcox.test(abs(perc.Verschil) ~ binbui, data=data20noNA, na.rm=TRUE, paired=FALSE,
exact=FALSE, conf.int=TRUE)

print(m1)

#Hodges Lehmann Estimator (hoeveel meer of minder te verwachten)

m1$estimate

# Volgens de Mann-Whitney U test is er dus een significant verschil

# tussen binnen en buiten, waarbij het de afwijking tov het Neubauer gemiddelde

# in het binnen-oppervlak lager is dan dat van de buitenrand

#####

# Dan voor onder vs boven

#####

#Descriptive statistics maken van de dataset

data20noNA %>% select(bovond, perc.Verschil.BovOnd) %>% group_by(bovond) %>%
summarise(n = n(),
          mean = mean(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          sd = sd(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          stderr = sd/sqrt(n),
          LCL = mean - qt(1 - (0.05 / 2), n - 1) * stderr,
          UCL = mean + qt(1 - (0.05 / 2), n - 1) * stderr,
          median = median(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          min = min(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          max = max(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          IQR = IQR(abs(perc.Verschil.BovOnd), na.rm = TRUE),
          LCLmed = MedianCI(abs(perc.Verschil.BovOnd), na.rm=TRUE)[2],
          UCLmed = MedianCI(abs(perc.Verschil.BovOnd), na.rm=TRUE)[3])

# Boxplot maken om te kijken naar eventuele outliers

ggplot(data20noNA, aes(x = bovond, y = abs(perc.Verschil), fill = bovond)) +
  stat_boxplot(geom = "errorbar", width = 0.5) +
  geom_boxplot(fill = "light blue") +
  stat_summary(fun.y=mean, geom="point", shape=10, size=3.5, color="black") +

```

```

ggtitle("Objective 20X") +
theme_bw() + theme(legend.position="none") +
xlab("Location") +
ylab("% deviance relative to Neubauer conc. (x100)")

# Dan testen op normaliteit
data20noNA %>% group_by(bovond) %>%
  summarise(`W Stat` = shapiro.test(abs(perc.Verschil.BovOnd))$statistic,
            p.value = shapiro.test(abs(perc.Verschil.BovOnd))$p.value)

# p-waardes voor zowel de normale als de absolute dataset is <0.05
# dus data is niet normaal verdeeld

# Met een qq-plot kun je dit visualiseren
ggplot(data = data20noNA, mapping = aes(sample = abs(perc.Verschil.BovOnd), color = bovond, fill =
bovond)) +
  stat_qq_band(alpha=0.5, conf=0.95, qtype=1, bandType = "boot") +
  stat_qq_line(identity=TRUE) +
  stat_qq_point(col="black") +
  facet_wrap(~ bovond, scales = "free") +
  labs(x = "Theoretical Quantiles", y = "Sample Quantiles") + theme_bw()

# Ook in qqplots dus duidelijk niet normaal verdeeld, datapunten
# zouden de lijn moeten volgen, is niet het geval

# Daarom dus Mann-Whitney-U test uitvoeren (Wilcox-test)
m2<-wilcox.test(abs(perc.Verschil.BovOnd) ~ bovond, data=data20noNA, na.rm=TRUE,
paired=FALSE, exact=FALSE, conf.int=TRUE)
print(m2)

#Hodges Lehmann Estimator (hoeveel meer of minder te verwachten)
m2$estimate

# Ook hier een significant verschil volgens de MWU-test

```

```
# De afwijking tov de Neubauer gemiddeldes is lager in de onderste helft
# van het preparaat tov de bovenste helft
```

```
#####
```

```
# ANALYSE VOOR 40X VERGROTING
```

```
#
```

```
#####
```

```
# BINBUI
```

```
data40 <- read.csv2("SA_locatie_40.csv", header = TRUE)
```

```
#Descriptive statistics maken van de dataset
```

```
data40 %>% select(BinBui, perc.Verschil) %>% group_by(BinBui) %>%
```

```
  summarise(n = n(),
```

```
    mean = mean(abs(perc.Verschil), na.rm = TRUE),
```

```
    sd = sd(abs(perc.Verschil), na.rm = TRUE),
```

```
    stderr = sd/sqrt(n),
```

```
    LCL = mean - qt(1 - (0.05 / 2), n - 1) * stderr,
```

```
    UCL = mean + qt(1 - (0.05 / 2), n - 1) * stderr,
```

```
    median = median(abs(perc.Verschil), na.rm = TRUE),
```

```
    min = min(abs(perc.Verschil), na.rm = TRUE),
```

```
    max = max(abs(perc.Verschil), na.rm = TRUE),
```

```
    IQR = IQR(abs(perc.Verschil), na.rm = TRUE),
```

```
    LCLmed = MedianCI(abs(perc.Verschil), na.rm=TRUE)[2],
```

```
    UCLmed = MedianCI(abs(perc.Verschil), na.rm=TRUE)[3])
```

```
# Boxplot maken om te kijken naar eventuele outliers
```

```
ggplot(data40, aes(x = BinBui, y = abs(perc.Verschil), fill = BinBui)) +
```

```
  stat_boxplot(geom = "errorbar", width = 0.5) +
```

```
  geom_boxplot(fill = "light blue") +
```

```
  stat_summary(fun.y=mean, geom="point", shape=10, size=3.5, color="black") +
```

```
  ggtitle("Objective 40X") +
```

```

theme_bw() + theme(legend.position="none")+
xlab("Location") +
ylab("% deviance relative to Neubauer conc. (x100)")

# Dan testen op normaliteit
data40 %>% group_by(BinBui) %>%
  summarise(`W Stat` = shapiro.test(abs(perc.Verschil))$statistic,
            p.value = shapiro.test(abs(perc.Verschil))$p.value)

# p-waardes voor zowel de normale als de absolute dataset is <0.05
# dus data is niet normaal verdeeld

# Met een qq-plot kun je dit visualiseren
ggplot(data = data40, mapping = aes(sample = abs(perc.Verschil), color = BinBui, fill = BinBui)) +
  stat_qq_band(alpha=0.5, conf=0.95, qtype=1, bandType = "boot") +
  stat_qq_line(identity=TRUE) +
  stat_qq_point(col="black") +
  facet_wrap(~ BinBui, scales = "free") +
  labs(x = "Theoretical Quantiles", y = "Sample Quantiles") + theme_bw()

# Ook in qqplots dus duidelijk niet normaal verdeeld, datapunten
# zouden de lijn moeten volgen, is niet het geval

# Daarom dus Mann-Whitney-U test uitvoeren (Wilcox-test)
m3<-wilcox.test(abs(perc.Verschil) ~ BinBui, data=data40, na.rm=TRUE, paired=FALSE, exact=FALSE,
conf.int=TRUE)

print(m3)

#Hodges Lehmann Estimator (hoeveel meer of minder te verwachten)
m3$estimate

# Volgens deze MWU-test is er dus geen significant verschil te zien tussen
# afwijking van het gemiddelde tussen de binnen of buitenkant van het
# preparaat

```

```
#####

# BOVOND

#####

#Descriptive statistics maken van de dataset

data40BovOnd <- data40 %>% select(BovOnd, perc.Verschil.BovOnd)
data40noNA <- data40BovOnd %>% filter(rowSums(is.na(data40BovOnd)) == 0)

data40noNA %>% select(BovOnd, perc.Verschil.BovOnd) %>% group_by(BovOnd) %>%
  summarise(n = n(),
            mean = mean(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            sd = sd(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            stderr = sd/sqrt(n),
            LCL = mean - qt(1 - (0.05 / 2), n - 1) * stderr,
            UCL = mean + qt(1 - (0.05 / 2), n - 1) * stderr,
            median = median(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            min = min(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            max = max(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            IQR = IQR(abs(perc.Verschil.BovOnd), na.rm = TRUE),
            LCLmed = MedianCI(abs(perc.Verschil.BovOnd), na.rm=TRUE)[2],
            UCLmed = MedianCI(abs(perc.Verschil.BovOnd), na.rm=TRUE)[3])

# Boxplot maken om te kijken naar eventuele outliers

ggplot(data40noNA, aes(x = BovOnd, y = abs(perc.Verschil.BovOnd), fill = BovOnd)) +
  stat_boxplot(geom = "errorbar", width = 0.5) +
  geom_boxplot(fill = "light blue") +
  stat_summary(fun.y=mean, geom="point", shape=10, size=3.5, color="black") +
  ggtitle("Objective 40X") +
  theme_bw() + theme(legend.position="none")+
  xlab("Location") +
  ylab("% deviance relative to Neubauer conc. (x100)")

# Dan testen op normaliteit
```



```

data40noNA %>% group_by(BovOnd) %>%
  summarise(`W Stat` = shapiro.test(abs(perc.Verschil.BovOnd))$statistic,
            p.value = shapiro.test(abs(perc.Verschil.BovOnd))$p.value)

# p-waardes voor zowel de normale als de absolute dataset is <0.05
# dus data is niet normaal verdeeld

# Met een qq-plot kun je dit visualiseren

ggplot(data = data40noNA, mapping = aes(sample = abs(perc.Verschil.BovOnd), color = BovOnd, fill =
BovOnd)) +
  stat_qq_band(alpha=0.5, conf=0.95, qtype=1, bandType = "boot") +
  stat_qq_line(identity=TRUE) +
  stat_qq_point(col="black") +
  facet_wrap(~ BovOnd, scales = "free") +
  labs(x = "Theoretical Quantiles", y = "Sample Quantiles") + theme_bw()

# Ook in qqplots dus duidelijk niet normaal verdeeld, datapunten
# zouden de lijn moeten volgen, is niet het geval

# Daarom dus Mann-Whitney-U test uitvoeren (Wilcox-test)

m4<-wilcox.test(abs(perc.Verschil.BovOnd) ~ BovOnd, data=data40noNA, na.rm=TRUE,
paired=FALSE, exact=FALSE, conf.int=TRUE)

print(m4)

#Hodges Lehmann Estimator (hoeveel meer of minder te verwachten)

m4$estimate

# Volgens deze MWU-test dus net geen significant verschil tussen
# afwijking van Neub. gemiddelde van telling in de bovenste
# of onderste helft van het preparaat

```

## Appendix 6: R-script for analysis CASA-dataset

```
IVFdata <- read.csv2("IVF_data_CASA.csv", header = TRUE)

attach(IVFdata)

library(dplyr)

IVFcompact <- IVFdata %>%
  select(Conc_voor, VCM_voor, VCM_voor_range, VCL_voor, VCL_na,
         VSL_voor, VSL_na, VAP_voor, VAP_na, ALH_voor, ALH_na, BCF_voor, BCF_na,
         WOBB_voor, WOBB_na, NrOva, X2PN, X0PN, NrEmb, Zwanger.Binair,
         Leeftijd.t.t.v..Behandeling) %>% filter(NrOva >= 3)

#####
# Variabele maken met percentage bevruchte eicellen
#####

attach(IVFcompact)

IVFcompact$perc.Bevr <- (X2PN/NrOva)

#####
# NA's er uit filteren
# Columns van Zwanger.binair, X2PN, X0PN en NrEmb er ook uithalen
#####

new_IVFcompact <- IVFcompact[rowSums(is.na(IVFcompact)) == 0,]
new_IVFcompact <- select(new_IVFcompact, -c(Zwanger.Binair, X2PN, X0PN, NrEmb))

#####
# Analyse
#####

str(IVFcompact)

IVFcompact$Zwanger.Binair <- as.factor(IVFcompact$Zwanger.Binair)

object <- glm(perc.Bevr~., data = new_IVFcompact)
lm_obj <- lm(perc.Bevr~., data = new_IVFcompact)

summary(lm_obj)
```

```

summary(object)

#####

# Grafieken maken general linear modelling

#####

library(ggplot2)

ggplot(new_IVFcompact, aes(x = Conc_voor , y = perc.Bevr)) +
  geom_point() +
  stat_smooth(method = "lm", col = "red") +
  ylab("% fertilized oocytes (x100)") +
  xlab("Concentration before processing (*10^6 per ml)")

ggplot(new_IVFcompact, aes(x = NrOva , y = perc.Bevr)) +
  geom_point() +
  stat_smooth(method = "lm", col = "red") +
  ylab("% fertilized oocytes (x100)") +
  xlab("Number of oocytes harvested")

ggplot(new_IVFcompact, aes(x = ALH_na , y = perc.Bevr)) +
  geom_point() +
  stat_smooth(method = "lm", col = "red") +
  ylab("% fertilized oocytes (x100)") +
  xlab("Amplitude of lateral head displacement (uM per sec)")

#####

#Zwanger.Binair analyseren, logistic regression

#####

new2_IVFcompact <- IVFcompact[rowSums(is.na(IVFcompact)) == 0,]
new3_IVFcompact <- dplyr::select(new2_IVFcompact, -c(X2PN, X0PN, NrEmb))
mylogit <- glm(Zwanger.Binair ~ ., data = new3_IVFcompact, family = "binomial")
summary(mylogit)

```

```
#####

# Grafiek maken logistic regression

#####

ggplot(new3_IVFcompact, aes(x=Conc_voor, y=as.numeric(Zwanger.Binair) - 1)) +
  geom_point(alpha=.5) +
  stat_smooth(method="glm", se=FALSE, fullrange=TRUE,
             family = binomial) +
  xlim(-10, 650) +
  ylab("Pregnancy (0 = no, 1 = yes)") +
  xlab("Spermatozoa concentration before processing (*10^6 per ml)")

ggplot(new3_IVFcompact, aes(x=perc.Bevr, y=as.numeric(Zwanger.Binair) - 1)) +
  geom_point(alpha=.5) +
  stat_smooth(method="glm", se=FALSE, fullrange=TRUE,
             family = binomial) +
  xlim(-0.1, 1.1) +
  ylab("Pregnancy (0 = no, 1 = yes)") +
  xlab("% fertilized oocytes (x100)")
```