

A meta-analysis of clinical pathology results from South African bred Sprague Dawley rats

By

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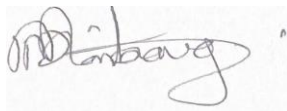
DECLARATION

The work reported in this dissertation was carried out in the Section of Pharmacology and Toxicology of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Professor Vinny Naidoo.

The mini-dissertation has not been submitted at this University or any other academic institution for the purpose of obtaining another qualification. It is the result of my own work, except where acknowledged I, Mr. Merapelo Duke Maribeng, and declare the statements above to be correct.

Ethics statement: The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval. The author declares that he/she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

Mr. Merapelo Duke Maribeng



Prof. Vinny Naidoo (Supervisor)

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ABSTRACT

Rats are laboratory animals that play a pivotal role in pre-clinical toxicity testing. There are two major classes of rats available for this exercise, inbred and outbred stocks. Inbred stocks are well known for their genetic uniformity, which normally results in less variation phenotypically when responding to pharmacological effect as opposed to outbred stocks during pre-clinical toxicity trials. Despite these animals being derived and classified, they are not resistant to genetic drift, which can influence the design, results and reproducibility of the said animal model. While genetic drift may not result in major changes in the genetic characteristics of the animals, it can also have more insidious changes in the clinical pathology of the animals. Since South Africa represents a small breeding colony, we undertook a retrospective study for this study to ascertain if there is a risk of genetic drift existing in the Sprague Dawley rats.

A meta-analysis of the mean parameter values obtained from four previously conducted independent studies was undertaken. Statistical heterogeneity was interpreted as the variability of pathological parameters across the four studies considered. The I^2 statistic was used to describe the percentage of variation across the studies that is due to heterogeneity rather than chance. To determine whether statistical heterogeneity is greater than it would be by chance, a chi-squared test of the hypothesis of heterogeneity of effects using Cochrane's Q statistic was conducted. Random effects models were a natural choice based on the assumption that toxicity levels (via measured parameters) were not identical but follow some distribution. The model represents a lack of knowledge about why real, or apparent, genetic drift effects differ across the studies by considering the differences as if they were random.

From the meta-analysis the 90% confidence intervals were determined and compared to the reference intervals available from Charles River laboratories, a major rodent breeder in the world. There were variations for parameter across the three main categories of white blood cells, red blood cells and serum chemistry. While some variables did fall within the published reference intervals, there were many deviations. For instance, absolute monocyte counts were higher than the normal ranges for both genders while mean corpuscular haemoglobin concentration and haematocrit had lower values than normal ranges. While the animals selected for this analysis are known to be healthy, and also confirmed on pathology, these post mortem findings supports our concerns of genetic drift being present and interfering with clinical pathology values. This has implications on future toxicology studies conducted on inbred SD rats and the interpretation of results, as the Charles River published reference intervals are unlikely to be relevant to South Africa. It is thus recommended that the results determined by meta-analysis should be validated or a completely new reference interval be established in these SD rats as per recommended statistical methodology for determining clinical pathology reference intervals.

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LIST OF ACRONYMES

RDW:	Red cell distribution Width
WCC:	White Cell Count
Neuts (mat) :	Neutrophils (matured) percentage
N (immat) %:	Neutrophils (immature) percentage
Lymph %:	Lymphocytes percentage
Mono %:	Monocytes percentage
Eos %:	Eosinophils percentage
Baso %:	Basophils percentage
N (mat) abs:	Absolute neutrophils (mature) count
Lymph abs:	Absolute neutrophils(immature) count
Mono abs:	Absolute monocytes count
Eos abs:	Absolute eosinophils count
Baso abs:	Absolute basophiles count
Plt C:	Platelet count
MPV:	Mean Platelet Volume

Aniso:	Anisocytosis (Unequal blood cell)
Polychr:	Polychromasia
Plt smear:	Platelet smear
Ht:	Haematocrit
HPLC:	High Performance Liquid Chromatography
MCH:	Mean corpuscular haemoglobin
TP:	Total protein
ALB:	Albumin
GLOB:	Globulin
A/G ratio:	Albumin-Globulin ratio
ALT:	Alanine amino transferase
ALP:	Alkaline phosphatase
GGT:	Gamma-glutamyl transferase
GLUC:	Blood glucose
Na ²⁺ :	Sodium
K ⁺ :	Potassium
CHOL:	Cholesterol
UREA:	Urea
CREAT:	Creatinine
UPBRC:	University of Pretoria Biomedical Research Centre
F1:	First filial generation
F2:	Second filial generation
CPOE	Computerized physician order entry
GCP	Good Clinical Practice
MCI	Medical Council of India
SAD	Single Ascending Dose
SD	Sprague Dawley
MAD	Multiple Ascending Dose

MTD	Maximum tolerated dose
API	Active Pharmaceutical Ingredient
IND	Investigational New Drug
GMP	Good Manufacturing Practices
DS	Drug Substance
GLP	Good laboratory Practices
VCG	Visual Central Groups

Glossary

Homeopathy: It is a system of alternative drug usage where diseases are treated with potentised liquids which in large quantities are able to cause disease symptoms.

Allopathy: A traditional way of disease treatment.

Ayurveda: It is the Hindu traditional system of medicine where a balance of bodily systems is involved and uses yogic breathing and the use of herbs.

Plasma: It's the liquid part of blood that does not contain blood cells from cells and but rich in proteins.

Serum: It is the liquid part of blood after clotting.

Allele: It is another form of a gene in a rat which may occur in pairs.

Bottle Neck Genetic Drift: a form of genetic drift where large numbers of rat population is reduced.

Derivatization: A process by which a genetic population is changed to include an organism that has genetic properties suitable to a certain analytical method.

Excipients: An inactive substance that serves as a vehicle of a drug.

LD₅₀: It is a lethal dose of a drug substance which when given at once causes 50 % death of test animals.

Medicine: It is a process that involves the processes prevention, diagnosis, treatment and health support to patients.

Chapter 1: Introduction

1.1 Background

Laboratory rats play a pivotal role in pre-clinical toxicity testing. There are two major classes of rats available for such research, being inbred and outbred stocks. Inbred stocks are well known for their genetic uniformity, which normally results in less variation phenotypically when responding to pharmacological effects as opposed to outbred stocks during pre-clinical toxicity trials. The use of inbred rats is favored as there is an increased statistical power with reproducibility being improved resulting in the use of fewer animals during trials. In contrast it has also been argued that outbred rats due to their genetic variability, are more predictive of a genetically heterozygous human population. As a result, numerous models of laboratory animals, which are supported by extensive genetic information, have been developed to support testing. (Jensen et al., 2016).

When undertaking toxicity studies, the evaluation of the study results is based on a set protocol for which clinical pathology variables are evaluated. For said clinical pathology results, it is also important that the evaluated variables are within a given range and are tightly regulated through statistical methodology. When there is a deviation from these reference “normal” intervals, be it an increase or a decrease in their concentration, a pathologic or unexpected activity is likely occurring. Variables commonly monitored are blood cell counts (hematology), coagulation factors and serum chemistry (e.g. Calcium, sodium, chloride, globulins, albumin, bicarbonate and potassium). With clinical pathology variables linked to toxicity studies commonly submitted to government regulatory departments, it has also been suggested that they need to be harmonized (Everds et al., 2016 and Weingand et al., 1992).

Following the elucidation of the study results, the usual practice is to compare the results to historical control data, as a way to verify the toxicological and safety findings of the pharmaceutical test material. However, for this standardized approach towards interpretation and description of clinical pathology variables to function, requires the results to be compared to a normal clinical pathology variable from a comparable strain (Hall et al., 2017).

Despite the use of defined and classified rodent strain in toxicity testing, it should be noted that animals are not immune to genetic drift (changes in genetic and phenotypic characteristics as the animal's generations breed away from their originator parents. These spontaneous mutations may be caused by errors during DNA replication and meiosis, deletion, duplications, inversions and single nucleotide polymorphisms (SNP). (Janine Low-Marchelli., 2017). Furthermore, Brekke et al., 2017 and Shimoyama et al., 2017 indicates that while genetic drift generally does not result in major changes in the genetic characteristics of the animals, it can have more insidious changes in the clinical pathology of the animals, which can influence the design, results and reproducibility when using the said animal model. A change in genetic characteristics influences clinical pathology evaluations, therefore the variables also change, these variables include coagulation, hematology and serum chemistry and their variation can also be influenced gender and age.

With Janine Low-Marchelli., 2017 suggesting that genetic drift cannot unfortunately be completely eliminated, particularly in actively breeding rats, it is therefore important to implement genetic stability in both small and large colony establishments. In large breeding centers, mitigation of the changes induced by genetic drift is done through regular genetic testing and restocking of laboratory animals in a form of derivatization. It can further be stated that for smaller breeding sites, such as South Africa, it is not always easy to restock, nor is it cost effective. More importantly, no information is available to describe the degree of genetic drift from South African bred rodents in comparison to the progenitor stock available internationally.

Based on studies undertaken at the University of Pretoria's controlled laboratory animal center, there has been some concerns as four studies completed one yearly over four years, with Sprague Dawley rats from the same breeder, they all had variables such as Ht and MCH which fell outside the reference intervals described internationally. To ensure optimal interpretation of the clinical pathology results from toxicity studies completed locally, it would be important to know the reference intervals for these rats. It would also be important to know if the results differ significantly from the reference intervals described internationally. For this study, the results from the four mentioned studies were evaluated through meta-analysis to ascertain the likely local reference intervals for comparison to the international published reference interval. If a difference

is noted, it would indicate that the reference interval be established for the locally bred strain of Sprague Dawley (SD) rats.

1.3 Problem statement

Some South African bred rats have their clinical pathology variables are no longer in the specified reference intervals published internationally for the same (originator) strain of rat, and are suspected to have succumbed to genetic drift.

1.4 Study aim

This study aimed to indirectly assess the presence of genetic drift of Sprague Dawley rats based on clinical pathology variables from preclinical toxicity studies in animals from the same source.

1.5 Objectives

To examine clinical pathology variables of Sprague Dawley (SD) rats from pre-clinical toxicity testing studies conducted at the the University of Pretoria Biomedical Research Centre (UPBRC) and use a meta-analysis method to create reference intervals per variable for the four studies, relevant to SD rats bred in South Africa.

Chapter 2: Literature Review

2.1 Impact of Medication in the World

When evaluating how the world manages to cope with health problems, it is evident that people are dependent on pharmaceutical agents for the treatment of different disease conditions, be it in human or veterinary medicine. There are different fields in medicine that people depend on according to their respective beliefs, and these include Homeopathy, Allopathy and Ayurveda amongst numerous others. It is important to note, that the utilization of traditional and modern medicines respectively, are approached with difference in their philosophy of approach. While traditional medicine is based on the use of herbal and non-pharmaceutical way of disease treatment, in contrast, modern medicine uses pharmaceutical agents in the form of vaccines and remedies, linked with advanced diagnostics. (More. B., 2016)

2.1.1 Dangers of allopathic medication

While there are tremendous benefits to the patient in using medicines or vaccines as mentioned above, this process is not entirely safe and can pose a danger to patients, by the involvement of side effects, that may range from mild, moderate, severe and eventually death. Numerous reasons can be responsible for side effects seen such as errors in the manufacturing process, with deviation from a reference or any applicable standard (Jain, 2017 et al and Jamie et al., 2016). These include and are associated with personnel who may be inadequately qualified and trained to execute their duties properly, poor equipment maintenance, and/or with non-adherence to its verification and calibration schedules. In addition, there can be errors associated with units of measurements, where there is no traceability of International System of Units (SI) to a natural constant, or an applicable primary standard (Jain, R K .,2017).

Factory environment with reference to its temperature, humidity, sterility and pressure are key elements which always has to be taken into account, which if not properly controlled can also be a source of a problem. Materials used in a pharmaceutical environment are of great importance and the methods used should always be validated so to assess their fitness to purpose. To overcome

these concerns, pharmaceutical manufacturers normally have a quality office, responsible for the implementation of quality policies for quality control and assurance, hence should be independent of the internal processes, thus to be impartial and report to top management (ISO 17025:2017).

Acheampong et al., 2014 has outlined that another major cause of side effects arise in hospitals from errors associated with personnel, where prescribing, dispensing and pharmaceutical administration is done incorrectly. Of these, prescription problems have been found to cause the most serious error and can result in more than just harm to the patient but can lead to death. Corrective action measures involved to this effect include, although not limited to, the implementation of the computerized physician order entry (CPOE) in hospitals. The CPOE can also be coupled with the clinical decision support tool (CDS) to increase the level of efficiency to avoid suboptimal prescribing and inadequate medication knowledge. The other reasons for drugs being dangerous, arises from the inherent dangers associated with the specific chemicals included in the final formulation. These dangers can be numerous and among others they include cellular toxicity, activation of non-target pathways, toxic metabolites and allergic reactions.

To mitigate against the inherent toxicity of a molecular substance, significant investigations are undertaken through a number of studies to ensure that chemicals or compounds selected as therapeutic agents are as minimally toxic as possible. Furthermore, while not all side effects can be prevented, mitigation strategies are also aimed at giving the treating doctor the best choice in managing a disease in terms of the risk-benefit analysis (Acheampong et al.,2014). In drug development, there are numerous steps in the development process. These include preclinical efficacy, pre-clinical toxicity and clinical testing. The preclinical efficacy studies are designed to determine the ability of a drug to have an effect; while preclinical toxicity is designed to determine the overt toxicity of a molecule, while clinical testing is designed to evaluate the efficacy of the final product in the target species under expected conditions of use. (Acheampong et al.,2014).

2.1.2 Drug Formulation

Alvi et al., 2009 explained that drug formulation involves mixing the Active Pharmaceutical Ingredient (API) with other chemical substances known as excipients, into a form that allows for proper administration by a defined route of administration and absorption by a defined route such as parental, enteral, topical, or through inhalation. The API is the substance which is intended for use in the pharmaceutical development of a medicine and become an active ingredient, be able to cause disease prevention, cure and affect functioning and structure of the body. APIs are developed by processes including recombinant DNA technology, fermentation, and chemical synthesis. APIs are required for in vivo dose studies, early formulation and efficacy studies.

The excipients are the inactive substances in the formulation and serve as a vehicle of a drug (Edward., 2009). When formulations are compounded, considerations are given to physical and chemical compatibility of the components, and may have dissociation enhancers to improve the bioavailability of the drug substance (DS). Normally a simple formulation is prepared as the drug product used in the clinical studies. Formulation analysis allows to identify contamination with impurities and verifies the final product purity, identity and physicochemical properties. The process of drug formulation also involves verification of inactive and active compounds, dissolution properties, assessment of potency and dosage.

2.1.3 Pre-clinical testing

A number of tests have been designed to evaluate the preclinical safety of the drugs prior to their entry into veterinary or medical use. When tests are undertaken, the requirement is for one rodent and non-rodent species to be used in battery of defined tests. There is also quantification of a dose, which is implemented in the form of efficacy and potency (Brodniewicz and Gronkiewicz, 2010).

2.1.4 Clinical testing

Throat et al. (2010) indicates that clinical trials involve an investigation of new pharmaceutical agents in people or the final target animal subject. The transition from pre-clinical to clinical trials remains a tool that determines an introduction of a new pharmaceutical agent in the market. There are different types of clinical trials which may include therapeutic trials, which involves testing of new combinations of products or new approaches such as radiation or surgery approaches. There may also be prevention trials responsible for investigating the ability of a substance in preventing disease and include vaccines, drugs to be used in prophylaxis, minerals and vitamins. Diagnostic trials are performed to identify the most suitable diagnostic products and procedures for a condition or a disease. Screening tests are exceptional to certain infections and health conditions.

2.1.5 Toxicity testing

This is a mandatory process enforceable by the prevailing laws of a regulator where humans and animals are going to be exposed to pharmaceutical agents in the environment. This toxicity can be determined by clinical studies, this involves studies in animals (in vivo), and laboratory studies (in vitro), and lastly through an epidemiology study which involves a cohort, case control, cross sectional (ecological study) (Committee of Toxicity and Carcinogenicity (2021). Report of the Synthesis and Integration of Epidemiological and Toxicological Evidence Subgroup (SETE)).

- **Cohort studies:** occurs when animals or humans exposed to a pharmaceutical agent are followed over time prospectively.
- **Case-control study:** this is a retrospective comparison of individuals who have been exposed to a pharmaceutical agent with similar control subjects.
- **Cross-sectional studies:** involves studying of a disease prevalence in an exposed group.
- **Ecological studies:** it is when the existence of a disease in one area due to a pharmaceutical exposure is compared with an incidence in another area where there is no hazardous pharmaceutical agent.

In vivo tests are carried out in animals and the following should be taken into consideration; dosage and frequency, novelty, physico-chemical properties, specie and sex of the animal. Tests normally carried out are acute, it involves one dose, and sub-chronic where a dose is repeated after 28-90 days and chronic where a dose is repeated over one year. These tests include ecotoxicity, carcinogenicity, teratogenicity and reproductive toxicity (Commission Implementing Decision (EU) 2020/569).

Acute tests are responsible for determining dose levels, drug substance toxicity and subsequent determination of adverse effects. Sub-chronic and chronic studies are purposed to indicate toxicity on target organs, and pathological effects. In-vitro tests are based on the philosophy of replacement, reduction, and refinement. Replacement means animals should be replaced with alternatives. Reduction involves the adoption of measures to reduce the number of animals used, and the refinement of methods used to achieve a correct animal welfare and distress reduction. As an example these tests include and are not limited to derma toxicity which involves the use of skin cells, ocular toxicity where tests are performed on an excised post mortal eye and not in the whole animal, and genotoxicity by the use of Ames test for detecting mutations in bacteria e.g. *Salmonella typhimurium* (Timbrell, 2001).

2.1.6 Bio analytical Methods in Pharmacological Testing

Edward (2009) and Everds (2015) states that irrespective of the type of study undertaken, an important aspect in drug testing is to determine the exact changes in physiology. In most cases, this involves the analysis of physiological fluids, such as plasma, blood and urine for induced changes and when necessary to determine drug substance amounts in the test animal. For the biological testing of samples, an evaluation of blood components, being red blood cells, white blood cells, and platelets are important. Testing can also include the impact on the homeostatic system, and include inflammation markers like fibrinogen, white blood cells and serum proteins.

Ordinarily clinical pathology measurements that are regulated are within a defined health range. An increase or decrease in these variables normally results from the presence of a pathological process. Commonly regulated parameters include uric acid, coagulation factors, blood cell counts,

globulins, albumin, sodium, chloride, bicarbonate, potassium, and calcium. For an altered homeostasis these parameters will therefore serve as markers. Meanwhile there are also some variables that are not regulated because their variations do not directly affect a health condition, and thus rather exist as markers for an underlying condition (Everds, 2015). These latter measures may further be adaptive or injury related. These include, but are not limited to, liver function enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine amino transferase (ALT), and gamma glutamyl transferase (GGT). With consideration that these parameters generally indicate a response to a metabolic change, they rather correlate with structural changes detectable by microscopy, and are less known to correlate with clinical changes. (Everds, 2015).

When evaluating the effects that a drug substance can have on the physiological parameters being monitored, it is not only important to determine if change has occurred but also important it is the extent of the change. Therefore the results are typically compared between groups such as dosing groups or comparisons between control and test groups, with interpretation of data including descriptive statistics where the data is screened for normality, box plots, and panel graphs (Hartmann et al., 2020). Another model of key importance in evaluating the changes per dose group is to also compare the results to a virtual control groups (VCGs), through the use of historical control data. There has also been a recommendation for replacement of control group animals with historical data for over some decades now to minimise animals used in research. While the use of virtual control groups is attractive due to the ethical implications of smaller animal numbers and reduced costs of tests, the use of a virtual control groups requires historic study data to be converted into reference intervals. Reference intervals are created by the use of a non-parametric method, at a confidence interval of 90 % when a minimum of 120 reference values are available. The fractiles serve as lower and upper limits at 2.5th and 97.5th respectively (Hartmann et al., 2020 and Friedrichs et al., 2006).

While the use of reference intervals has major advantages, there are a number of factors that can impact on the clinical pathology parameters:

- Use of inbred or outbred strains: During toxicity studies there is the choice of using either an inbred or outbred strain. Inbred strains are normally of great preference, they have been

identified to be genetically stable and uniform allowing for repeatability to be easily attained during trials. Procedures done using these strains results in fewer false negative due to consistency in the model. Also, since the animals are very similar, there is no reason to increase the number of animals involved in a study. In contrast outbred strains are more genetically undefined with animals being genetically unknown and unique. In toxicology studies, instead of inbred species, outbred stocks are preferred as they have the same variation that is present in the target human or veterinary population. Not surprisingly, the reference intervals generated for a particular strain is different and thus not interchangeable between the strains (Festing et al., 2010).

- Age of animal: A rapid stage of growth such as adolescence have been known to influence physiological changes hence rats of this age have been considered for studies. Certain effects also occur due to age differences. It can be noted that these effects can be evident in studies of longer duration or in short studies, and are most notable to bone growth, hematologic system, and muscles mass. As an example, progressive decrease in reticulocytes counts is seen as the animals mature. It can also be noted that younger animals tend to have an increased neutrophil and lymphocyte counts (Aulbach et al., 2016).
- General strains of rats in use: Strains amongst others include Sprague-Dawley (SD), Wistar (Wi), and Long-Evans are typically used in toxicity studies. They have a diverse genetic composition hence allowing the study population to represent the heterogenic human population. As expected from different strains, they are all defined by slightly different clinical pathology variables (Karyn Colman., 2016).
- Concurrent disease occurrence: can exist during clinical trials, and has a potential to interfere with clinical pathology parameters. As an example, a tumour can result in lymphopenia and neutrophilia. It has been further indicated that bacterial endotoxins resulted in hypotension, hence the occurrence of an increased packed cell volume and decreased number of platelets, and monocytes (James et al., 2013).

Breeding age of the colony is fundamental in breeding, with breeders only being kept between 2 and 8 months of age, especially since older rats may not reliably breed. As a common practice a minimum of six breeding pairs that represent different generations in a colony are kept, and

maintenance of two generations of a strain. If a strain is used for more than ten generations this results in a genetic drift. There are age related phenotypes that has developed over time in commonly used rats of inbred strains. As an example, the C57BL/6J strain has an impaired glucose tolerance as a result of missense mutation resulting in the decline of mitochondrial function, and has implications for diabetic implications. Similarly in strain AKR/J, while continuing brother-sister mating over several years it started to show a decreased activity, coarse skin, hair loss and dull hair (Kevin Flurkey and Joanne Curren., 2009).

In consideration to the above mentioned, this research work will evaluate possibilities of genetic drift on previous studies in South Africa. More specifically, the Faculty of Veterinary Science of the University of Pretoria, has been running toxicity studies for a number of years. During these studies, some concerns have been noticed, in that the study animal clinical pathology values did not match the reference intervals published from a large international breeding colony. Since these studies used rats of the same strain, of the same age, mixture of gender and from an inbred colony, led to the speculation that genetic drift could be present.

2.1.7 Causes of Genetic Drift

A changed occurrence in the genetic makeup of a population active in breeding as a result of randomized mutations over time is regarded as genetic drift. The resultant new mutation is then incorporated in the breeding population until the allele or the population becomes extinct. Genetic drift may severely decrease the population size while on the other hand contribute to the formation of new population (Kevin Flurkey and Joanne Curren, 2009). The loci resulting from genetic drift in an enclosed population of breeding animals is influenced by the background rate of mutation, the number of animals involved in breeding that contributes to each generation, and the breeding scheme. The maintenance of the same breeding stock in random mating, results in an increased number of the segregating loci in an inbred strain, hence the fixation or extinction of new strains will be reduced. (Kevin Flurkey and Joanne Curren, 2009).

As mentioned above, since the genetic drift can result in a new strain being formed, it is important that for animals used in studies, for the background of the strain, its origin and the identification

of any mutations and proper quality control measures to be implemented. During intercrossing of two different strains, this allows an easy traceability exercise to the parent stock and it becomes easy to address health, welfare and proper colony maintenance is implemented. (Zimmerman et al., 2000)

2.1.8 Determination of Genetic Drift

Due to lower genetic differences in inbred stock, the following tools have been employed to determine the genetic make-up of an organism, due to the existence of a certain phenotype.

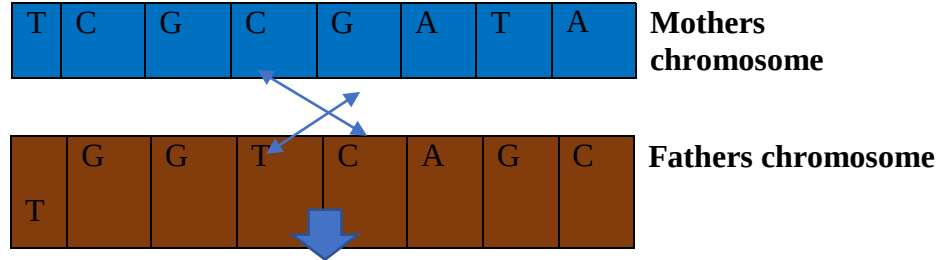
2.1.9 Linkage analysis

Kevin Flurkey and Joanne Curren., 2009 has noted that two phenotypic traits are considered to be linked together when they appear together in the same organism. Linkage traits group together to form linkage groups, and the frequency at which two traits link to each other, they are then considered to be close together, as opposed to when the traits are distributed independently, they are deemed to be from different linkage groups.

The genetic distance between the linked loci, which is a determinant of linked traits can be established by association between linked traits, thus calculating the genetic distance.

Benavides et al., 2019 denotes that the introduction of a genetic marker to each linkage, can help in the identification of a newly formed phenotype by studying its association with the genetic marker. By calculation of the genetic distance of a new phenotype, this can also locate the trait loci, resulting in a successful exercise of identifying genetic drift in a population (Figure 1).

Before Linkage



After linkage

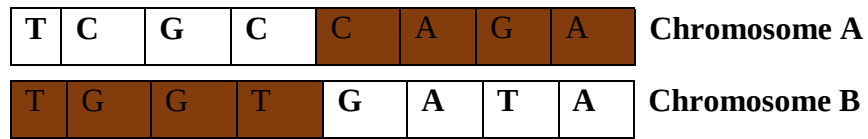


Figure 1 This figure shows the process of recombination. Homologous chromosomes of parents are represented in the upper DNA sequence, meanwhile the blue arrows show the point of where a genetic linkage took place within a chromosome. The lower DNA sequence shows a result of chromosome linkage, this is a genetic variation depicting a genetic drift and this can be transcribed into the individual's offsprings (Mijangos et al., 2017).

2.10 Genotyping

Genotyping is performed by the deoxyribose nucleic acid (DNA) analysis on rodent tissues. This is achieved using a polymerase chain reaction (PCR) where the least amount of DNA material is required. Ear punches, fecal samples, ear and mouth swabs can be used as samples for DNA analysis. Common markers mostly used for genotyping include among others, identification of single nucleotide polymorphisms (SNPs) and simple sequence length polymorphisms (SSLPs) whereby alleles are distinguished based on a single nucleotide difference within a short deoxyribose nucleic acid (DNA) sequence employed for this, and it is mostly useful for typing mice before breeding C.Bashur et al., 2015).

Biochemical markers are isoenzymes that can be used to compare proteins that possess different physical characteristics including enzymatic activity or electrophoretic mobility. Two strains may differ by 3-50 biochemical and immunological markers during analysis. Determination of characteristics are often derived from blood. These markers are quick, inexpensive, reproducible and simple to use. Markers such as “C” gene for coat color, while skeletal structure, reproductive performance, body size, are represented by the adrenocortecosteroid hormones, onset of disease and pathology can be identified by elevated number of cytokines and monocytes respectively. (Brancaccio et al., 2010)(Figure 2).

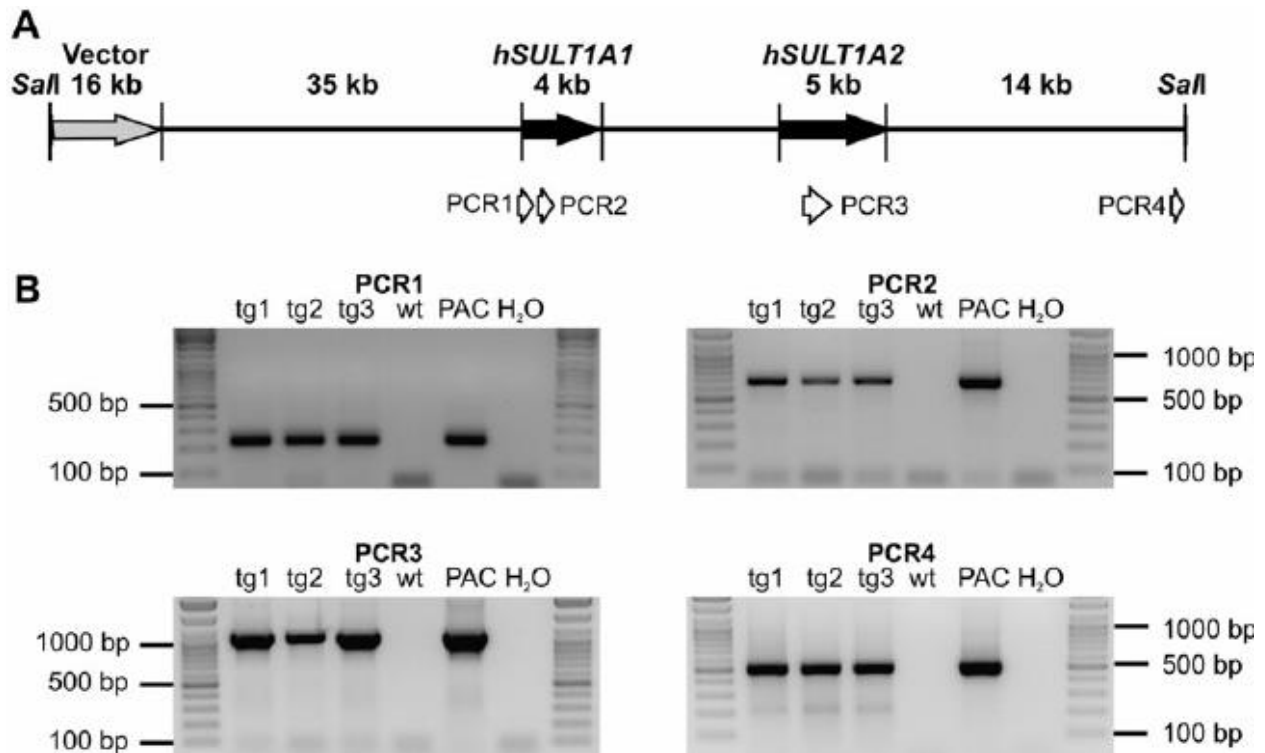


Figure 2: PCR used for genotyping hSULT1A1/1A2 -transgenic mouse lines. (A) An illustration of PCR scheme of the human PAC DNA used for microinjection (85 kb total). SalI, restriction site used for linearization. (B) Mouse tail DNA was amplified with primers for PCR1 (241 bp product), PCR2 (656 bp product), PCR3 (1094 bp product) and PCR4 (508 bp product). PCR products were separated on 1.5% agarose gels. tg1, tg2, tg3, mice of different hSULT1A1/1A2 -transgenic lines; wt, wild-type mouse; PAC, hSULT1A1/1A2-PAC DNA (positive control); H₂O, no DNA (Dobbernack et al., 2011).

2.11 Mapping

Genetic maps involve the calculation of meiotic recombination frequency between genomic markers so as to achieve the linearity and distance between loci. It analyses quantitative trait loci (QTL) linking disease phenotypes to gene intervals harboring disease modifiers. Its accuracy results in appropriate calculation of a QTL interval. This is because a trait linkage to a gene interval is a function of the genome distance between two markers. (Littrell et al., 2018).

When two non-identical markers are further apart from each other, this can result in the occurrence of multiple cross overs, as an example when holding a single crossover constant, changing the position of the second one results in two crossover meioses which are equal. This distance between markers is measured in centimorgans (cM), it quantifies the recombination frequency, which is equal to 1 % chance that a marker will be separated from another marker at a certain locus during a single generational cross over. Recombination hotspots exist in mammals hence the centimorgans do not correlate properly with the physical distances. Hotspots are numerous in a chromosome and differ within the inbred stock ((Miriam Schmid,2009). Figure 3 below shows a genetic mapping.

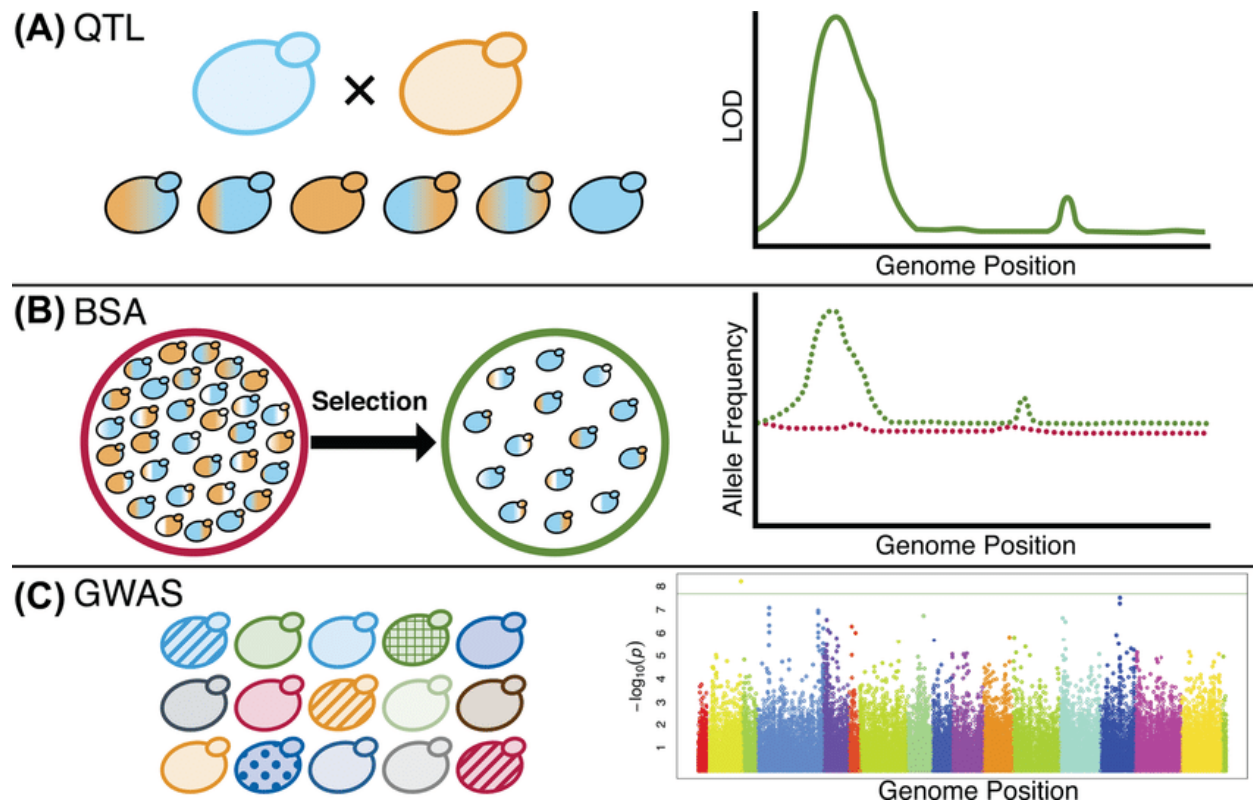


Figure 3: Examples of genetic mapping methods. (A) Quantitative trait loci (QTL) mapping crosses two strains with opposing phenotypes and interrogates genotype–phenotype correlations to implicate loci with high logarithm of odds (LOD) score. (B) BSA is an extension of QTL analysis in which millions of progeny are interrogated in a pool, such that alleles whose frequency increases in the post-selection pool are implicated as causal. (C). In contrast, GWAS compare genotype and phenotype across many individual strains to implicate loci with correlations unlikely to occur by chance (as indicated by $-\log$ of the p-value) (Gasch., 2017).

2.12 Minimizing the Effect of Genetic Drift

As evident above, one cannot stop genetic drift from occurring. The most effective way to minimize its impact, would be to perform periodic replacement of breeders with new stock rather than to use laboratory animals which were involved in breeding before, hence eliminating the possibility of divergence from a reference standardized breeding stock. This is best achieved by

cryopreservation of embryos in the same generation, and periodically replace breeders with these frozen stock, with subsequent lengthening of the generation time (Janine Low-Marchelli., 2017). It has been noted that the level of homozygosis in inbred strains is not absolute, some degree of heterozygosis will always prevail due to spontaneous mutations. The steady state level of heterozygosis can be affected by the strategies involved in breeding.

As a result, it is important to employ strict usage of sister-brother mating in a single lineage so as to, increase the elimination rate of new mutations. Sister-brother mating reduces the steady state of heterozygosis, and maximizes fixation of new mutations because they have similar genetic linearity. Implementation and maintenance of the Genetic Quality Control (GQC) and Genetic Stability Program (GSP) can also be employed to reduce genetic drift as outlined below (Janine Low-Marchelli.,2017).

2.13 Maintenance of Genetic Quality Control

The Genetic Quality Control (GQC) and Genetic Stability Program (GSP) exist to minimize genetic contamination and the effects of genetic drift, according to a specified standard operating procedure. For Genetic Stability Program (GSP), it assures practically that the number of generations is within seven generations from the main pedigreed phase by using cryopreservation. To support such a programme requires a live repository to be a place.

It comprises of the standard inbred strains to be used, but will also include other strains such as mutants, genetically engineered strains, and potential newly imported strains. Cryopreservation also needs to be practiced and cryopreserved stocks include sperm, ova and embryo and a backup facility is always. The backup storage facility serves as an instrument to recover strains from frozen material and generation of live mice (Zimmerman et al.,2000)

For the programme, sufficient embryos are frozen for each foundation for a single generation that last for 10-25 years, while its re-establishment is done at each and every five generations. This

programme extends the length of a generation by ten to twenty-five years, hence at 100 years the foundation stock will have advanced at no more than 4-10 generations. This is known to retard the rate of genetic drift by twenty to fifty-fold in short lived species (Janine Low-Marchelli., 2017). Colonies can then be divided into three tiers, so as to maintain a pedigreed stock of inbred strains, thereby maximizing genetic consistency and reducing genetic drift effects. These tiers are separated from each other and they include foundation, pedigreed expansion, and production stocks. With this structure we are able to identify an animal with undesirable genetic traits and remove it from the breeding population. Foundation stocks (FSs) maintenance is through sister-brother mating.

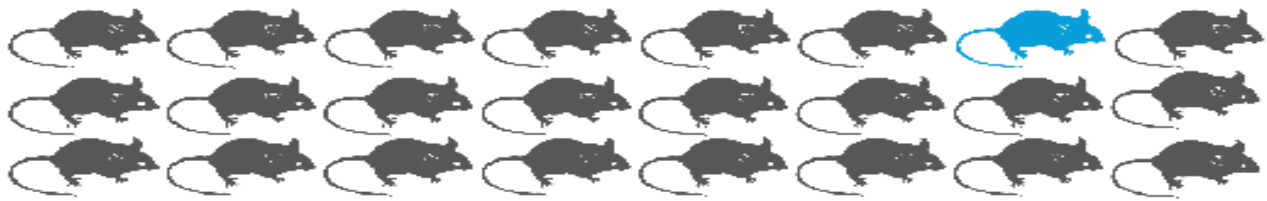
It is common to maintain one family per strain, meanwhile when strains are in high demand, one can use two families (Zimmerman et al.,2000). These families are never crossed and are maintained in such a way that they are never more than ten generations from an inbred pair of sister-brother, hence preventing development of accidental sub-strains.

Embryos from foundation stocks are cryopreserved for derivation, in case the stock is lost. Pedigreed expansion stocks (PESs) are a composition of pedigreed sister-brother pairing derived from the foundation stocks. PESs are responsible for expanding foundation stock thus creating breeders for the production stock (Janine Low-Marchelli.,2017).

To achieve rapid expansions, sibling trio mating can be employed, involving two sisters and one brother to minimize genetic drift. It is mandatory to make sure that PESs are maintained at less than five generations after post removal from the foundation stock. Production stocks (PSs) compose breeding pairs originating from PESs and their offspring, this is the mice that is commonly distributed for studies by researchers. Production stocks are known to never exceed two generations regardless of the expansion method and mice from foundation stock should not exceed seven generations (Zimmerman et al.,2000). Figure 4 below shows the incidence and impact of crossing small and large colonies

A

Large Colony



Small Colony



B

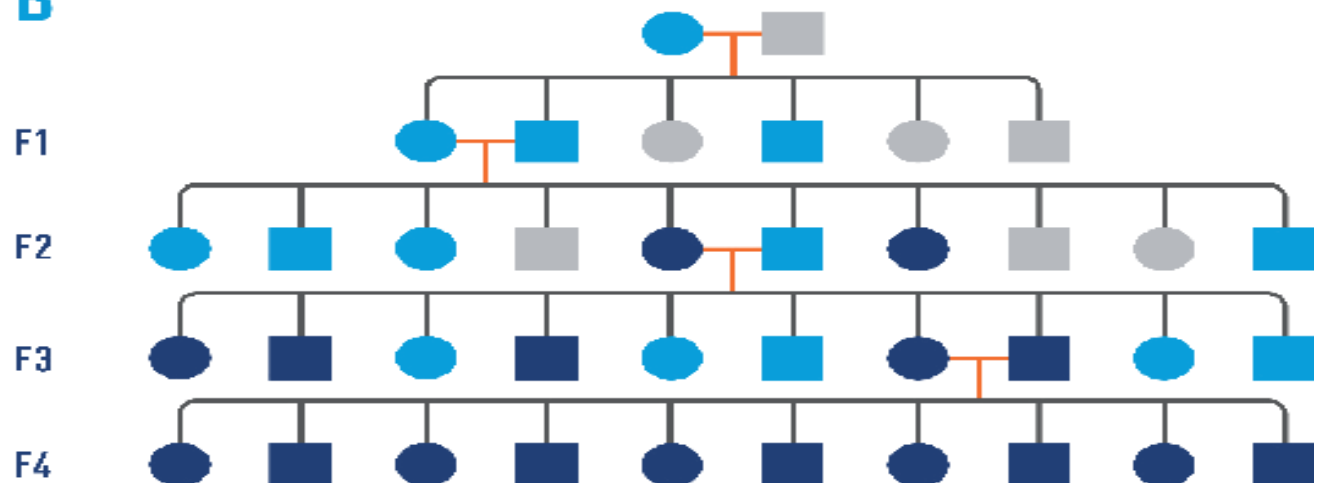


Figure 4: The risk of propagating a spontaneous mutation is higher in small colonies versus large colonies. A) The probability of using a mouse that carries any given mutation (light blue) for breeding is higher in a small colony than a large colony. B) In each round of breeding, there is a 25% chance that a new mutation will become more established in the population. For example, Mendelian inheritance predicts that the F1 generation will be composed of 50% wildtype (grey) and 50% heterozygous for the mutation (light blue). If by chance, two heterozygotes are used as breeders, the F2 generation will be composed of 25% wildtype, 50% heterozygotes, and 25% homozygotes (dark blue). This can continue until the entire colony is fixed homozygous for the mutation (F3, F4). However, the genome can drift in either direction depending on the genotypes of the mice used for breeding – the probability that the mutation becomes fixed is equivalent to the probability it will be lost entirely from the colony. (Janine Low-Marchelli., 2017).

CHAPTER 3: Methodology

3.1 Primary studies

This study utilized previously collected clinical pathology data from four studies on Sprague Dawley (SD) rats that took place in different years. The studies in question were all 90 days sub chronic toxicity investigation conducted on behalf of private pharmaceutical companies to determine the effects of four different pharmaceutical agent(s). The studies followed OECD toxicity guidelines and each consisted of four dosing groups where three groups were treated with the test substance at escalating doses, while the fourth group were the healthy control. In each of the four studies, the healthy controls SD rats were dosed sterile water for 90 days, by oral gavage. As at the time of writing this research report, none of the results from the four studies had been published in peer reviewed scientific journals. Due to confidentiality reasons, the specific details about the studies are not going to be discussed in this document. Table 3.1 below is a summary table of the duration and sample sizes of the studies.

Table 3. 1: Summary of the study characteristics of the four studies.

Study	Study period	Study design	Age (weeks)	Sample size		
				Males	Females	Total
BP4	2012	Cohort	>17	10	10	20
H25	2009	Cohort	>17	10	10	20
T022	2015	Cohort	>17	10	10	20
V0-29	2015	Cohort	>17	10	10	20

3.2 Study site

These animal studies were all conducted at the University of Pretoria Biomedical Research Centre (UPBRC) of the Faculty of Veterinary Science, University of Pretoria located in the north of Pretoria, in South Africa. The UPBRC has conducted multiple laboratory toxicity studies since its inception in 1994. All studies used the same methodology and personnel involved in facilitation of the studies.

3.3 Study Design

This study is a retrospective cohort study.

3.4 Study Population

The study population consisted of 80 SD rats that constituted the control groups from the four studies described in section 3.1 above.

3.5 Sampling

No sampling was done in this study, and only retrospectively collected data was evaluated from the samples that were collected and analyzed from the four studies described in table 3.1 above.

3.6 Data Collection

Laboratory results for the clinical pathology parameters of the SD rats were used in this study. The results for the four studies were evaluated by two laboratories, the Faculty's in-house laboratory (BP4 and H25) and the commercial IDEXX laboratory (Study TO-22 and VO-29). In both cases the results were evaluated on automated equipment that include centrifuges and automated analysers (Specific equipment details were not available for the meta-analysis), which was

validated for small mammal use. The variables evaluated include white blood cells, red blood cells and serum chemistry and were compared to international reference intervals depicted in Table 3.2.

Table 3.2: Description of pathological variables and Charles River reference intervals

Category	Parameter	Acronym	Units	Charles River Intervals	
				Males	Females
White blood cells	White blood cell count	WCC	10e9/l	1.98-11.06	0.96-7.88
	Neutrophils	Neuts	%	9-49.3	8.8-43.8
	Lymphocytes %	Lymph %	%	44.7-87.1	48.9-88.1
	Platelets	Plt	$C \times 10e9 / l$	574-1253	599-1144
	Eosinophils	Eos	%	0.4-4	0.3-4.7
	Neutrophils absolute	N	$abs \times 10e9 / l$	9-49.3	0.15-1.11
	Monocytes	Mono	%	1-3.6	1-3.6
Red blood cells	Red Cell Count	RCC		7.62-9.99	7.16-9.24
	Haemoglobin	HB	g/l	13.6-17.4	13.7-17.2
	Haematocrit	HT	l/l	38.5-52	38.5-49.20
	Mean Corpuscular volume	MCV	fl	46.3-56.2	50.3-57
	Mean Corpuscular haemoglobin	MCH	g/dl	16.3-19.5	17.6-20.3
	Mean Corpuscular haemoglobin Concentration	MCHC	g/dl	31.9-38.5	32.2-37.80
	Red Cell Distribution Width	RDW	%	11.6-16.2	10.6-14.6
Serum chemistry	Albumen	ALB	g/dl	3.6-4.7	3.7-5.8
	Alanine amino transferase	ALT	u/l	19-48	14-64
	Creatinine	Creat	mmol/l	0.3-0.5	0.3-0.6

3.7 Data Analysis

Results from each study were converted to a mean value and a reference interval and then subsequently compared to the published reference interval available from Charles River Laboratories, a major breeder of SD rats internationally as outlined in table 3.2 above. Descriptive statistics were used to provide a summary of each laboratory parameter studied. Specifically, the mean ($\pm$ standard deviation), range, median and interquartile range were reported for each parameter.

A meta-analysis of the mean parameter values was conducted and results presented using forest plots for a consolidated reference interval (Higgins et al., 2019). The 90% confidence intervals were used as the reference intervals for the pooled estimates obtained for the meta-analysis. These were calculated as $pooled\ mean \pm 1.64 \times \frac{SD}{\sqrt{n}}$ where 1.64 is the standard normal value for 90% CI, SD is the standard deviation of the pooled mean and n is the number of observations for each parameter.

In this study, statistical heterogeneity was interpreted as the variability of pathological parameters across the four studies considered. The I^2 statistic was used to describe the percentage of variation across the studies that is due to heterogeneity rather than chance. A general guide to the interpretation of the I^2 statistic that was used in this study is presented in **Table 3.3** below.

Table 3.3: Interpretation of the Heterogeneity categories.

I^2 Category	Interpretation
0% to 40%	Not important
30% to 60%	Moderate heterogeneity
50% to 90%	Substantial heterogeneity
75% to 100%	Considerable heterogeneity

In this analysis, the hypothesis used to test for heterogeneity was;

H_0 : There is no difference in pathological parameter values across the 4 studies reviewed.

H_1 : There is a difference in pathological parameter values across the 4 studies reviewed.

Higgins et al., 2019 states that to determine whether statistical heterogeneity is greater than it would be by chance, a chi-squared test of the hypothesis of heterogeneity of effects using Cochrane's Q statistic should be conducted. For this part of the analysis, all the clinical pathology parameters obtained from the toxicity part of the study were used (n=320, from 16 dosing groups). The study made use of a random effects model based on the assumption that even when the differences were due to toxicity levels (via measured parameters) they would follow some form of distribution. The model thus represented a lack of knowledge about why real, or apparent, toxicity effects differ across the studies by considering the differences as if they were random. Statistical heterogeneity was determined at a cut-off value of p-value <0.05 whereas the contrary was determined using p-values above this cutoff (p-value ≥ 0.05). The 90% confidence intervals were used for this study to provide a wider reference range considering to account for a small sample used (Table 3.1).

3.8 Ethical Approval

This study was approved by the Research Ethics Committee (*REC250-19*) of the University of Pretoria.

CHAPTER 4: Results

This chapter outlines results of the data analysis process described in section 3.7 above. Descriptive statistics of clinical pathology variables are first presented followed by results on meta-analysis of the same parameters. Results on descriptive statistics and meta-analysis presented in this chapter are outlined in three thematic areas, namely; white blood cells, red blood cells and serum chemistry as previously outlined in table 3.2 above.

4.1 Descriptive statistics

4.1.1 White Blood Cells

The lowest mean count of WCC was observed in study V0-29 at $3.57 \times 10^9/L$ while the highest was observed on study BP4 at $19.7 \times 10^9/L$. Study T022 and V0-29 had relatively higher mean counts of WCC ($\times 10^9/L$) at 8.64 ($SD: \pm 1.89$) and 8.58 (± 2.01) respectively, compared to the mean WCC counts obtained from both BP4 and H25 7.38 (± 3.35) and 7.34 (± 1.79) respectively. A similar pattern of higher mean WCC counts in studies T022 and V0-29 were observed across gender compared to studies BP4 and H25.

Neutrophils (%) were reported from three of the four studies with the exception of study T022. Study BP4 had both the lowest (4%) and highest (93%) neutrophil values in percentage which was observed among the female SD rats. The overall mean percentage neutrophil values ranged from 14.65% (± 5.4) in study VO-29 to 19.78% (± 18.83) for study BP4. However, this mean value for study BP4 was affected by the outlier value in study BP4, this outlier mean value however is not indicated in the results summary. Among the three studies with neutrophils (%) values, the overall median value was lowest in study VO-29 (12.0, IQR: 8.0-16.0) and highest in study H25 (16.65, IQR: 13.8-20.1).

The widest range of lymphocytes (%) was observed among male rats in study H25 from 2.4 to 83.1%. Studies BP4 and H25 had relatively lower values of lymphocytes (%) at 79.45% (± 8.52) and 73.95% (± 17.72) compared to studies T022 and VO-29 whose overall mean values were 82.75 (± 4.93) and 80.55 (± 5.83) respectively. Narrow differences were observed in the median values of lymphocytes (%) across gender in each of the four studies with no identifiable outlier values observed. Results for WCC, neutrophils (%) and lymphocytes (%) are shown in table 4.1 below.

Table 4.1: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of white blood cells parameters WCC, neutrophils and lymphocytes.

Study	Sex	WCC			Neuts			Lymph (%)		
		Mean (SD)	Range	Median (IQR)	Mean	Range	Median	Mean (SD)	Range	Median (IQR)
					(SD)		(IQR)			
BP4	Females	7.14 (4.59)	3.77-19.70	5.53 (5.08-7.27)	20.53 (25.87)	4.00-93.00	13.85 (10.00-16.00)	83.48 (5.)	76.00-92.00	83 (79.4-86.30)
	Males	7.62 (1.6)	4.08-10.02	8.12 (6.81-8.35)	19.04 (8.84)	12.60-41.10	16.2 (13.30-19.90)	75.42 (9.6)	52.00-83.00	78.1 (74.20-82.40)
	Both	7.38 (3.35)	3.77-19.70	7.05 (5.34-8.20)	19.78 (18.83)	4.00-93.00	15.75 (12.30-19.40)	79.45 (8.52)	52.00-92.00	81.6 (76.35-83.50)
H25	Females	6.22 (1.79)	3.73-9.86	6.34 (4.87-7.09)	15.35 (2.35)	12.60-19.30	15.25 (13.10-17.20)	72.76 (24.79)	2.40-83.10	79.6 (78.90-82.20)
	Males	8.45 (.88)	7.01-9.43	8.44 (8.18-9.25)	19.93 (5.79)	12.70-29.00	19.45 (14.20-23.40)	75.14 (6.15)	64.00-82.20	75.8 (72.50-81.20)
	Both	7.34 (1.79)	3.73-9.86	7.39 (6.34-8.84)	17.64 (4.9)	12.60-29.00	16.65 (13.80-20.05)	73.95 (17.62)	2.40-83.10	78.95 (74.35-81.55)
T022	Females	8.18 (2.45)	4.67-13.05	8.62 (6.67-9.33)	0	0.00-0.00	0	85.59 (2.71)	80.00-90.00	85.45 (84.00-87.00)
	Males	9.1 (1.04)	8.12-11.33	8.55 (8.38-9.88)	0	0.00-0.00	0	79.91 (5.1)	70.00-87.90	80 (78.00-80.00)
	Both	8.64 (1.89)	4.67-13.05	8.55 (8.24-9.35)	0	0.00-0.00	0	82.75 (4.93)	70.00-90.00	84 (80.00-87.00)

Study	Sex	WCC			Neuts			Lymph (%)		
		Mean (SD)	Range	Median (IQR)	Mean	Range	Median	Mean (SD)	Range	Median (IQR)
					(SD)		(IQR)			
VO-29	Females	7.89 (2.31)	3.57-12.62	7.9 (6.89-8.75)	13.5 (6.93)	7.00-30.00	12 (8.00-16.00)	82.3 (7.24)	65.00-89.00	82.5 (80.00-88.00)
	Males	9.27 (1.45)	6.99-11.68	8.94 (8.74-9.61)	15.8 (3.26)	12.00-24.00	15 (14.00-16.00)	78.8 (3.52)	70.00-83.00	80 (79.00-80.00)
	Both	8.58 (2.01)	3.57-12.62	8.74 (7.41-9.32)	14.65 (5.4)	7.00-30.00	15 (11.00-16.00)	80.55 (5.83)	65.00-89.00	80 (80.00-84.00)

A summary of platelets and monocytes is shown in table 4.2 below. The overall mean platelet count was lowest in study T022 at $571.85 (\pm 197.01) \times 10^9/L$ while study BP4 reported the highest overall mean values of $834.03 (\pm 248.3) \times 10^9/L$. Similarly, the highest median values were observed in study BP4 at 887 (IQR: 756-980) $\times 10^9/L$ while the lowest median values were recorded at 614 (IQR: 445-723) for T022. Generally, a wide variation was observed in the mean platelet counts in each study across gender of the SD rats.

In addition, female rats had lower platelet counts across all studies except for study H25 where females had the highest mean platelet counts ($694 \times 10^9/L$) compared to males ($666 \times 10^9/L$). The lowest and highest platelet counts at $9.6 \times 10^9/L$ and $1114 \times 10^9/L$ respectively were recorded in female SD rats of study BP4.

Table 4.2: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of white blood cells parameters; platelets (Plt) and monocytes (Mono).

Study	Sex	Plt			Mono (%)		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	819.86 (343.58)	9.60- 1114.00	927.50 (738.00- 1069.00)	3.15 (1.93)	0.00-6.00	3.15 (2.00-4.00)
	Males	848.20 (108.02)	665.00- 996.00	841.50 (774.00- 955.00)	3.51 (0.92)	2.40-4.90	3.15 (2.80-4.50)
	Both	834.03 (248.30)	9.60- 1114.00	887.00 (756.00- 980.50)	3.33 (1.48)	0.00-6.00	3.15 (2.75-4.25)
H25	Females	694.30 (218.21)	268.00- 949.00	759.00 (606.00- 876.00)	2.64 (1.88)	1.30-7.90	2.10 (1.90-2.50)
	Males	666.00 (246.24)	119.00- 925.00	701.00 (665.00- 842.00)	3.52 (1.54)	2.30-7.00	2.85 (2.60-3.60)
	Both	680.15 (226.91)	119.00- 949.00	732.00 (627.50- 844.50)	3.08 (1.73)	1.30-7.90	2.50 (2.10-3.15)
T022	Females	525.10 (231.87)	198.00- 773.00	570.00 (268.00- 772.00)	3.25 (0.80)	2.00-4.10	3.20 (3.00-4.00)
	Males	618.60 (152.71)	249.00- 792.00	654.50	4.19 (1.06)	2.80-6.00	4.05 (3.00-5.00)

Study	Sex	Plt			Mono (%)		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
				(590.00-695.00)			
	Both	571.85 (197.01)	198.00-792.00	614.00 (445.50-723.00)	3.72 (1.03)	2.00-6.00	4.00 (3.00-4.10)
VO-29	Females	596.50 (198.15)	171.00-802.00	681.00 (583.00-723.00)	2.90 (0.99)	2.00-5.00	3.00 (2.00-3.00)
	Males	632.20 (128.29)	318.00-758.00	648.50 (612.00-728.00)	4.00 (0.67)	3.00-5.00	4.00 (4.00-4.00)
	Both	614.35 (163.50)	171.00-802.00	674.00 (583.50-725.50)	3.45 (1.00)	2.00-5.00	3.50 (3.00-4.00)

Summary statistics for eosinophils and absolute neutrophils counts are presented in table 4.3 below. The widest range (0-2.7%) of eosinophils was observed in study H25 while studies BP4 and T022 had a range of 0-2% each and study V0-29 ranged from 1-2%. The lowest mean eosinophils value was observed in study T022 at 1.12 % (± 0.45), followed by study BP4 at 1.2% (± 0.69), V0-29 at 1.35 (± 0.49) and H25 at 1.39 (± 0.70).

Male SD rats generally had higher mean eosinophils values compared to female rats for all studies except study H25 where males had a mean of 1.35 (± 0.79) versus female SD rats which had a mean 1.44 (± 0.65). The lowest median eosinophils values were observed in studies T022 and V0-29 at 1% (IQR: 1-1.1) and 1% (IQR: 1.0-2.0) respectively. In addition, the median eosinophils value for study BP4 was 1.35% (IQR: 1-1.8) and that of study H25 was highest of all studies at 1.4% (IQR: 0.95-1.8).

The mean absolute neutrophils count ranged from $1.24 (\pm 0.5) \times 10^9/L$ in study V0-29 to $1.42 (\pm 1.2) \times 10^9/L$ in study BP4. Study BP4 had the widest range of absolute neutrophils ($0.22-4.85 \times 10^9/L$), followed by study V0-29 with a range of $0.57-2.31 \times 10^9/L$ and study H25 whose range was $0.64-2.68 \times 10^9/L$. The median absolute neutrophils count ($\times 10^9/L$), in descending order, 1.21 (IQR: 0.88-1.38) for VO29, 1.18(0.82-1.57) for H25 and 1.10 (0.64-1.55) for BP4.

In figure 4.1, a panel of box plots for seven white blood cells parameters is presented. These graphs are a pictorial representation of tables 4.1-4.3. The values of the WBC parameters were fairly symmetrically distributed across the studies with a few instances of outliers observed. Relatively low mean parameter values were observed in study T022 for neutrophils, EOS and absolute neutrophils.

Table 4.3: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of the white blood cells parameters eosinophils and absolute neutrophils.

Study	Sex	EoS (%)			A Neut (%)		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	1.02 (0.91)	0.00-2.00	1.35 (0.00-1.80)	1.36 (1.48)	0.22-4.85	0.67 (0.55-1.57)
BP4	Males	1.37 (0.34)	1.00-1.90	1.35 (1.10-1.60)	1.47 (0.83)	0.68-3.53	1.18 (1.08-1.54)
BP4	Both	1.20 (0.69)	0.00-2.00	1.35 (1.00-1.75)	1.42 (1.17)	0.22-4.85	1.10 (0.64-1.55)
H25	Females	1.44 (0.65)	0.40-2.30	1.55 (1.00-1.90)	0.97 (0.40)	0.64-1.90	0.82 (0.72-1.08)
H25	Males	1.35 (0.79)	0.00-2.70	1.25 (0.90-1.60)	1.68 (0.57)	1.18-2.68	1.50 (1.18-1.89)
H25	Both	1.39 (0.70)	0.00-2.70	1.40 (0.95-1.80)	1.33 (0.60)	0.64-2.68	1.18 (0.82-1.57)
T022	Females	1.26 (0.41)	1.00-2.00	1.00 (1.00-1.40)	0.00 (0.00)	0.00-0.00	0.00 (0.00-0.00)
T022	Males	0.99 (0.47)	0.00-2.00	1.00 (1.00-1.00)	0.00 (0.00)	0.00-0.00	0.00 (0.00-0.00)
T022	Both	1.12 (0.45)	0.00-2.00	1.00 (1.00-1.10)	0.00 (0.00)	0.00-0.00	0.00 (0.00-0.00)
VO-29	Females	1.30 (0.48)	1.00-2.00	1.00 (1.00-2.00)	1.00 (0.46)	0.57-2.07	0.88 (0.61-1.19)
VO-29	Males	1.40 (0.52)	1.00-2.00	1.00 (1.00-2.00)	1.48 (0.44)	0.96-2.31	1.34 (1.23-1.74)
VO-29	Both	1.35 (0.49)	1.00-2.00	1.00 (1.00-2.00)	1.24 (0.50)	0.57-2.31	1.21 (0.88-1.38)

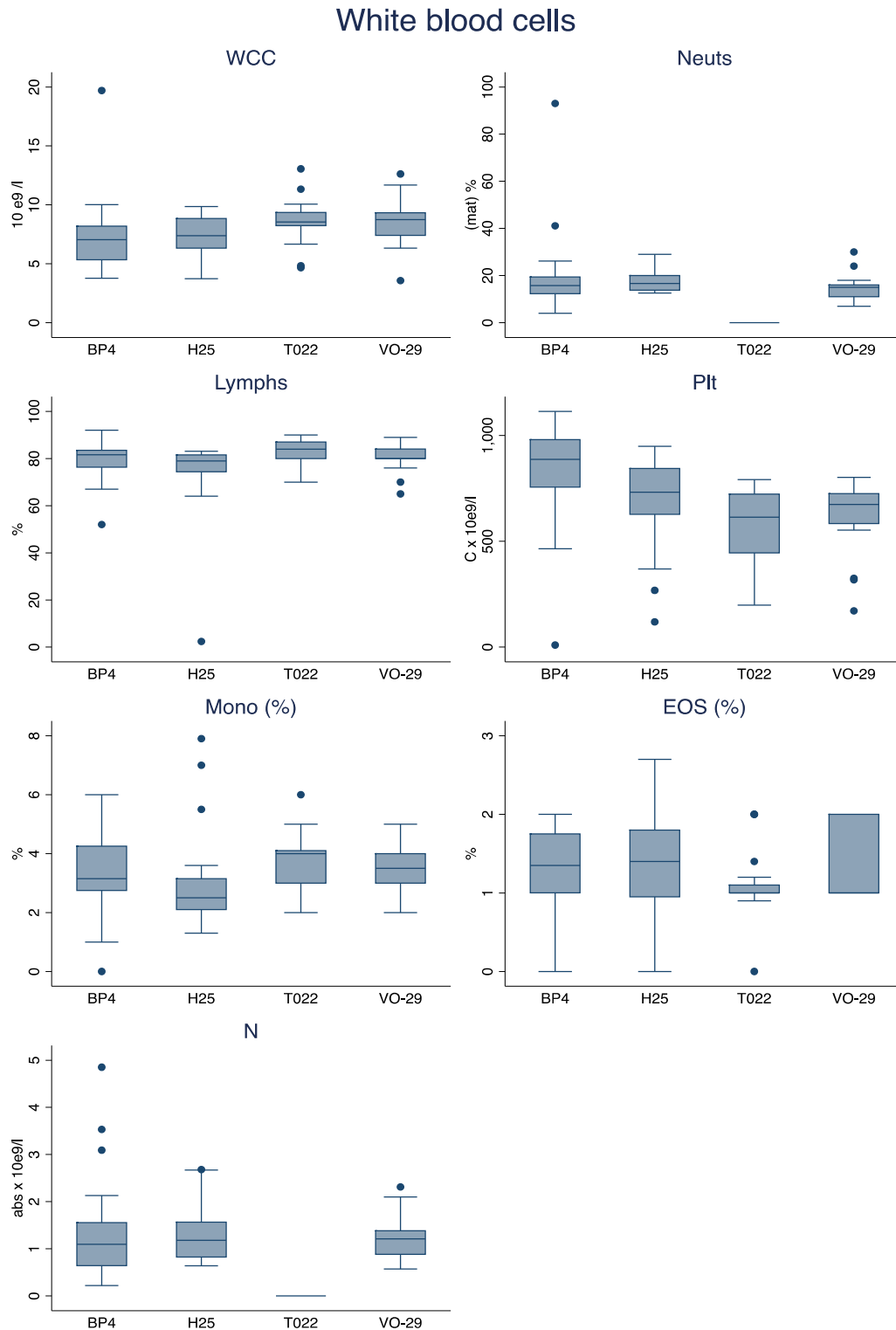


Figure 4. 1: Panel of box plots for white blood cells variables.

Key: **WCC**, White cell count, **Lymphs**; Lymphocytes, **Plt**; Plateletes **Neuts**; Immature neutrophils, **Mono**; Monocytes, **EOS (%)**; Eosinophiles percentage, **N**; Absolute Neutrophiles %; Eosinophile percentage

4.1.2 Red Blood Cells

The mean red blood cell counts were highest in study T022 at 40.40 (± 21.39) which was quite high in comparison to the other studies. In study BP4, this value was reported at 9.05 (± 0.86), in study H25 the mean was 8.34 (± 0.56) and 8.7 (± 0.68) for study V0-29. The high mean RCC value in study T022 could be attributed to presence of outliers. Specifically, this value ranged from 3-37 and 44-72 among female and male SD rats respectively. By comparison, the ranges of the other three studies were relatively narrow.

These ranges were 7.57-10.09, 7.53-9.34 and 7.58-9.67 for studies BP4, H25 and V0-29 respectively. Similarly, the RCC median values were highest in study T022 (40.50, IQR: 23-59.5) while the rest had median values of less than 10. The highest median RCC value of the three remaining studies was study BP4 (9.43, IQR: 8.17-9.76), followed by 8.36 (IQR: 7.87-8.82) and 8.95 (IQR: 8.04-9.24) for studies H25 and V0-29 respectively.

The overall mean HB values ranged from 15.23 (± 0.73) g/l in study H25 to 15.7 (± 1.11) in study BP4. The range of HB values varied from one study to the other. Study T022 had the widest range of 9.2-17.7 g/l, followed in descending order by BP4 (13.2-17.2 g/l), V0-29 (13.9-16.9 g/l) and H25 (14.1-16.4 g/l). The highest median Hb values were observed in study BP4 at 16.2 (IQR: 14.9-16.55), followed by V0-29 (15.9, IQR: 15.15-16.30), T022 (15.7, IQR: 15.15-16.25) and H25 (15.15 IQR: 14.65-15.95). Across all the four studies, male SD rats had higher mean Hb values compared to female rats in the same study. However, these differences were not very wide.

Table 4.4: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of red cell count (RCC) and Haemoglobin (Hb)

Study	Sex	RCC			HB		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	8.31 (0.55)	7.57-9.60	8.17 (8.11-8.41)	14.83 (0.89)	13.20-16.50	14.90 (14.40-15.30)
BP4	Males	9.78 (0.26)	9.26-10.09	9.76 (9.64-10.04)	16.57 (0.35)	16.10-17.20	16.50 (16.30-16.90)
BP4	Both	9.05 (0.86)	7.57-10.09	9.43 (8.17-9.76)	15.70 (1.11)	13.20-17.20	16.20 (14.90-16.55)
H25	Females	7.90 (0.30)	7.53-8.48	7.87 (7.69-8.05)	14.69 (0.35)	14.10-15.20	14.65 (14.50-14.90)
H25	Males	8.78 (0.37)	7.93-9.34	8.82 (8.71-8.90)	15.78 (0.57)	14.70-16.40	15.95 (15.40-16.20)
H25	Both	8.34 (0.56)	7.53-9.34	8.36 (7.87-8.82)	15.23 (0.73)	14.10-16.40	15.15 (14.65-15.95)
T022	Females	22.20 (11.48)	3.00-37.00	23.00 (14.00-33.00)	14.58 (2.03)	9.20-16.20	15.20 (14.70-15.60)
T022	Males	58.60 (9.89)	44.00-72.00	59.50 (52.00-67.00)	16.37 (0.76)	15.40-17.70	16.25 (15.90-17.20)
T022	Both	40.40 (21.39)	3.00-72.00	40.50 (23.00-59.50)	15.48 (1.75)	9.20-17.70	15.70 (15.15-16.25)
VO-29	Females	8.09 (0.35)	7.58-8.88	8.04 (7.91-8.26)	15.13 (0.69)	13.90-16.50	15.15 (14.80-15.50)
VO-29	Males	9.30 (0.22)	9.02-9.67	9.24 (9.16-9.47)	16.25 (0.37)	15.90-16.90	16.10 (15.90-16.60)
VO-29	Both	8.70 (0.68)	7.58-9.67	8.95 (8.04-9.24)	15.69 (0.79)	13.90-16.90	15.90 (15.15-16.30)

In table 4.5, summary statistics on haematocrit (Ht), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are presented. Study BP4 had the lowest Ht values ranging from 0.39-0.51 l/l and stratified by gender the range was 0.39-0.49 l/l among females and 0.42-0.51 l/l among males. The overall mean Ht values in this study was $0.45(\pm 0.03)$ and the median value was 0.45 (IQR: 0.43-0.48) l/l. Study H25 also had relatively low Ht values compared to the two remaining studies. In H25, the overall mean was 11.64 (± 19.9) l/l and the study had a very wide range of 0.40 to 47.60 l/l.

Male rats had a higher mean of 22.86 (± 23.64) and median of 22.03 (IQR: 0.46-45.3) compared to female rats whose mean and median Ht values were $0.42(\pm 0.02)$ and 0.42 (IQR: 0.41-0.43) l/l respectively. Ht values in studies T022 and V0-29 were 37.70-47.40 and 38.90-46-30 l/l respectively. In addition, studies T022 and V0-29 had higher overall mean values of $43.57 (\pm 2.38)$ and $43.60 (\pm 1.93)$, median values of 43.50 (IQR: 41.85-45.15) and 43.70 (IQR: 42.30-44.60) respectively.

There was little variation in the overall mean MCV values across the four studies and stratified by gender. Study T022 had the lowest mean value of $49.58 (\pm 2.77)$ fl, followed by V0-29, BP4 and H25 whose mean values were $50.32 (\pm 2.34)$, $51.20 (\pm 1.72)$ and $52.30 (\pm 1.48)$ respectively. In addition, the median values were 48.95 (IQR: 47.75-52.30), 49.60 (IQR: 48.35-52.85), 50.90 (IQR: 49.75-53.05) and 52.55 (IQR: 50.80-53.40) for studies T022, V0-29, BP4 and H25 respectively.

The mean corpuscular haemoglobin values were generally lower in studies T022 and V0-29 compared to BP4 and H25. The overall mean MCH values were $0.75 (\pm 1.13)$ and $1.81 (\pm 0.08)$ g/dl for studies T022 and V0-29 and median values of 0.37 (0.27-0.66) g/dl and 1.78 (IQR: 1.74-1.88) g/dl respectively. In addition, the observed ranges were 0.23-5.20 g/dl and 1.69-1.93 g/dl in studies T022 and V0-29 respectively. Studies BP4 and H25 had higher MCH values with overall means of $17.40 (\pm 0.69)$ and $18.30 (\pm 0.55)$, median values of 17.15 (IQR: 16.85-18.15) and 18.40 (IQR: 17.75-18.60) respectively. In addition, the MCH ranges for the two studies were 16.30-18.60 and 17.30-19.20 for BP4 and H25 respectively.

Table 4.5: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of white blood cells (WBC), haematocrit(Ht),mean corpuscular volume(MCV) and mean corpuscular haemoglobin (MCH)variables.

Study	Sex	Ht			MCV			MCH		
		Mean (SD)	Min – Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	0.43 -0.03	0.39-0.49	0.44 (0.42-0.45)	52.18 -1.81	47.90- 53.60	53.05 (51.50- 53.40)	17.85 -0.69	16.30- 18.60	18.15 (17.70- 18.20)
BP4	Males	0.47 -0.03	0.42-0.51	0.47 (0.45-0.49)	50.22 -0.91	49.20- 52.10	50.1 (49.50- 50.80)	16.94 -0.26	16.50- 17.40	16.9 (16.80- 17.10)
BP4	Both	0.45 -0.03	0.39-0.51	0.45 (0.43-0.48)	51.2 -1.72	47.90- 53.60	50.9 (49.75- 53.05)	17.4 -0.69	16.30- 18.60	17.15 (16.85- 18.15)
H25	Females	0.42 (0.02)	0.40-0.45	0.42 (0.41-0.43)	53.3 -1.24	50.10- 54.70	53.4 (53.30- 53.90)	18.6 -0.5	17.50- 19.20	18.6 (18.40- 19.00)
H25	Males	22.86 (23.64)	0.45- 47.60	22.03 (0.46-45.30)	51.3 -0.92	50.30- 53.10	50.9 (50.60- 52.00)	17.99 -0.43	17.30- 18.50	17.95 (17.70- 18.50)
H25	Both	11.64 (19.93)	0.40- 47.60	0.45 (0.42-22.03)	52.3 -1.48	50.10- 54.70	52.55 (50.80- 53.40)	18.3 -0.55	17.30- 19.20	18.4 (17.75- 18.60)
T022	Females	42.10 (2.00)	37.70- 45.20	41.85 (41.30- 43.20)	50.55 -3.22	44.00- 53.70	51.95 (47.80- 52.90)	1.22 -1.49	0.25-5.20	0.66 (0.49-1.05)

T022	Males	45.04 (1.77)	42.50- 47.40	44.8 (44.00- 46.40)	48.61 -1.94	45.80- 52.30	48.25 (47.10- 50.30)	0.29 -0.05	0.23-0.38	0.29 (0.24-0.31)
T022	Both	43.57 (2.38)	37.70- 47.40	43.5 (41.85- 45.15)	49.58 -2.77	44.00- 53.70	48.95 (47.75- 52.30)	0.75 -1.13	0.23-5.20	0.37 (0.27-0.66)
VO- 29	Femal es	42.25 (1.60)	38.90- 44.60	42.3 (41.50- 43.30)	52.22 -1.68	48.60- 54.10	52.85 (51.70- 53.30)	1.87 -0.05	1.74-1.93	1.88 (1.86-1.89)
VO- 29	Males	44.96 (1.11)	43.70- 46.30	44.6 (44.10- 46.10)	48.42 -0.82	46.90- 49.90	48.35 (48.00- 48.90)	1.75 -0.03	1.69-1.79	1.75 (1.73-1.77)
VO- 29	Both	43.60 (1.93)	38.90- 46.30	43.7 (42.30- 44.60)	50.32 -2.34	46.90- 54.10	49.6 (48.35- 52.85)	1.81 -0.08	1.69-1.93	1.78 (1.74-1.88)

The mean MCHC values did not vary substantially across studies and gender within the studies. These values, in ascending order, were 33.96 (± 0.42), 34.99 (± 0.52), 35.44 (± 3.13) and 35.94 (± 0.47) for BP4, H25, T022, and V0-29 respectively. In addition, the median values for each of the study were 33.90 (IQR: 33.60-34.30) g/dl, 34.95 (IQR: 34.80-35.30) g/dl, 35.95 (IQR: 35.65-36.10) g/dl and 36.10 (IQR: 35.85-36.25) g/dl for BP4, H25, V0-29 and T022 respectively. The widest range was observed in T022 (22.30-37.40 g/dl) while narrower ranges were observed in the remaining studies BP4 (33.20-34.90 g/dl), H25 (34.00-35.80 g/dl) and V0-29 (35.0-37.0 g/dl).

Study BP4 had the widest range of RDW (12.0-37.9%) was observed in BP4 while other ranges were 12-13.7%, 12.3-19.4% and 13.1-19.7% for H25, V0-29 and T022 respectively. The overall mean values were 12.8% (± 0.5), 15.25% (± 1.92), 16.28% (± 2.34) 16.32% (± 9.14) for studies H25, T022, V0-29 and BP4 respectively. On the contrary, BP4 and H25 had lower median values 12.65% (IQR: 12.4-13.0) and 12.85% (12.35-13.15) respectively compared to T022 and V0-29 whose median values were 15.14% (13.40-16.20%) and 16.40% (IQR: 13.95-18.40) respectively. The summary statistics for MCHC and RDW are presented in table 4.6 below.

The distribution of the red blood cells parameters was fairly symmetrical across the four studies with a few outliers observed. Study T022 had higher RCC values compared to the other 3 studies. Similarly, higher values of HT and RDW were observed for studies T022 and V0-029. MCH values were higher for studies BP4 and H25 compared to the other two studies. Figure 4.2 gives a pictorial presentation of the summary statistics for red blood cells parameters.

Table 4.6: Summary statistics showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of white blood cells parameters mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW)

Study	Sex	MCHC			RDW		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	34.19 (0.36)	33.60-34.90	34.30 (33.90-34.30)	19.88 (12.17)	12.00-37.90	12.40 (12.00-36.80)
BP4	Males	33.72 (0.35)	33.20-34.30	33.70 (33.60-33.80)	12.75 (0.22)	12.40-13.00	12.75 (12.60-13.00)
BP4	Both	33.96 (0.42)	33.20-34.90	33.90 (33.60-34.30)	16.32 (9.14)	12.00-37.90	12.65 (12.40-13.00)
H25	Females	34.90 (0.51)	34.10-35.70	34.90 (34.50-35.10)	12.46 (0.45)	12.00-13.60	12.35 (12.20-12.50)
H25	Males	35.08 (0.53)	34.00-35.80	35.05 (34.80-35.50)	13.13 (0.28)	12.60-13.70	13.10 (13.00-13.20)
H25	Both	34.99 (0.52)	34.00-35.80	34.95 (34.80-35.30)	12.80 (0.50)	12.00-13.70	12.85 (12.35-13.15)
T022	Females	34.61 (4.35)	22.30-36.90	36.10 (35.30-36.10)	15.25 (1.97)	13.10-19.70	15.15 (13.40-16.20)
T022	Males	36.26 (0.48)	35.80-37.40	36.15 (35.90-36.30)	15.25 (1.97)	13.10-19.70	15.15 (13.40-16.20)
T022	Both	35.44 (3.13)	22.30-37.40	36.10 (35.85-36.25)	15.25 (1.92)	13.10-19.70	15.15 (13.40-16.20)
VO-29	Females	35.80 (0.53)	35.00-37.00	35.70 (35.50-36.00)	14.14 (0.86)	13.30-16.00	13.95 (13.30-14.60)
VO-29	Males	36.09 (0.38)	35.60-36.80	36.05 (35.80-36.40)	18.42 (0.80)	16.80-19.40	18.40 (18.10-18.90)
VO-29	Both	35.94 (0.47)	35.00-37.00	35.95 (35.65-36.10)	16.28 (2.34)	13.30-19.40	16.40 (13.95-18.40)

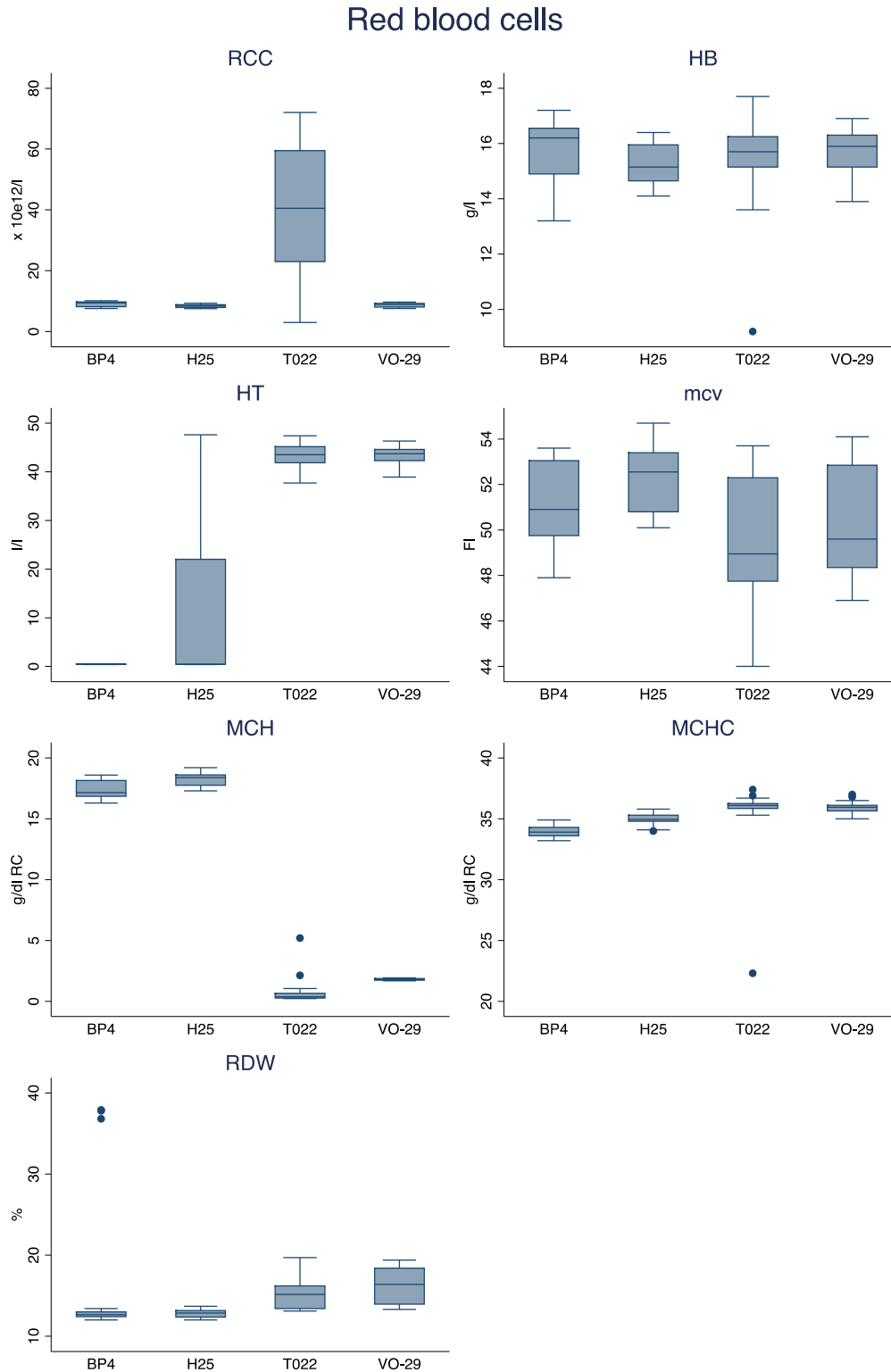


Figure 4. 2:Panel of box plots for red blood cells variables.

Key: **RCC**; Red cell count, **Hb**; Hemoglobin, **Ht**; Hematocrit, **MCV**; Mean corpuscular volume, **MCH**; mean corpuscular hemoglobin, **MCHC**; Mean corpuscular hemoglobin concentration, **RDW**; Red cell distribution width.

4.1.3 Serum Chemistry

Study BP4 had the highest ALB values for the overall mean (4.03 g/dl, SD=0.18), range of 3.64-4.14 g/dl and median (4.05g/dl, IQR: 3.94-4.14). The remaining three studies had mean ALB values of less than 4 g/dl. In study V0-29, the overall mean ALB was 3.61 g/dl (± 0.21) followed by H25 and T022 whose mean values were 3.45 g/dl (± 0.54) and 3.43 (± 0.38) g/dl respectively. The widest range of ALB was observed in study H25 (2.6-4.6 g/dl) followed by T022 (2.7- 4.2 g/dl), V0-29(3.2 – 4.1 g/dl) and BP5 (3.64 – 4.41 g/dl). The median ALB values, in descending order, were 3.27 (IQR: 3.10-3.77), 3.45 (IQR:3.30-3.60), 3.65 (IQR: 3.50-3.70) and 4.04 (IQR: 3.94-4.14) for studies H25, T022, V0-29 and BP4 respectively.

The range of ALT values were 24-168 u/l for BP4, 48-151 u/l for H25, 3-100 u/l in T022 and 32-39 u/l in study V0-29. The lowest mean ALT value was observed in female SD rats of study BP4 at 31.20 u/l (± 5.51). However, the other mean ALT values across gender and overall were considerably higher than the female rats of study BP4. The overall mean value ALT for study BP4 was 43.85 u/l (± 30.36), 67.10 u/l (± 21.90) for H25, 56.55 u/l (± 18.76) for T022 and 60.60 u/l (± 10.58) for V0-29. Similarly, study BP4 had the lowest median ALT value at 40 u/l (IQR:32.5-43.0), with increasing order at 54.5 u/l (IQR: 50.5-60.0), 61.5 u/l (IQR: 58.5-68.0) and 62.0 u/l (54.5-67.5) for studies T022, H25 and V0-29 respectively.

Studies BP4 and H25 had the lowest creatinine ranges (23.0-34.0 mmol/l) and (18.0-33.0 mmol/l) respectively, mean creatinine values at 27.90 mmol/l (± 3.43) and 24.35 mmol/l (± 3.33) respectively as well as the lowest median values at 27.50 mmol/l (IQR: 25.0-31.0) and 24.5 (IQR: 22.0-26.0) respectively. By comparison, higher values were observed in studies T022 (range: 54-82 mmol/l) and V0-29 (range: 51-109 mmol/l). The overall mean values were 62.75 mmol/l (± 7.28) and 68.5 mmol/l (± 12.55) for studies T022 and V0-29 respectively.

A wider range of ALB values were observed in study H25 compared to the rest. Quite a number of outliers were observed for all the three serum chemistry markers although distribution of data was fairly symmetrical across each study. Studies T022 and V0-29 reported the higher values of creatinine compared to studies BP4 and H25 (Figure 4.3).

Table 4.7: Summary showing the mean, standard deviation (SD), range (min-max), median and interquartile ranges (IQR) of serum chemistry variables albumin(ALB), alanine trans aminane (ALT) and Creatinine (Creat)

Study	Sex	ALB			ALT			Creat		
		Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)	Mean (SD)	Min - Max	Median (IQR)
BP4	Females	4.01 (0.24)	3.64-4.41	4.05 (3.82-4.16)	31.20 (5.51)	24.00-42.00	32.50 (26.00-34.00)	30.60 (2.55)	26.00-34.00	31.00 (29.00-33.00)
BP4	Males	4.04 (0.12)	3.83-4.23	4.05 (4.02-4.14)	56.50 (39.49)	39.00-168.00	43.00 (41.00-46.00)	25.20 (1.48)	23.00-28.00	25.00 (24.00-26.00)
BP4	Both	4.03 (0.18)	3.64-4.41	4.05 (3.94-4.14)	43.85 (30.36)	24.00-168.00	40.00 (32.50-43.00)	27.90 (3.43)	23.00-34.00	27.50 (25.00-31.00)
H25	Females	3.79 (0.51)	3.10-4.60	3.77 (3.34-4.10)	64.20 (12.05)	48.00-91.00	64.00 (58.00-68.00)	25.00 (4.14)	18.00-33.00	25.50 (22.00-27.00)
H25	Males	3.11 (0.31)	2.60-3.60	3.13 (3.00-3.20)	70.00 (29.12)	51.00-151.00	60.50 (59.00-68.00)	23.70 (2.31)	21.00-27.00	23.00 (22.00-26.00)
H25	Both	3.45 (0.54)	2.60-4.60	3.27 (3.10-3.77)	67.10 (21.90)	48.00-151.00	61.50 (58.50-68.00)	24.35 (3.33)	18.00-33.00	24.50 (22.00-26.00)
T022	Females	3.34 (0.37)	2.70-3.90	3.40 (3.20-3.60)	54.80 (9.44)	46.00-80.00	52.50 (50.00-56.00)	61.80 (5.43)	56.00-74.00	60.50 (58.00-62.00)
T022	Males	3.52 (0.39)	2.70-4.20	3.55 (3.40-3.70)	58.30 (25.44)	3.00-100.00	57.50 (51.00-66.00)	63.70 (8.97)	54.00-82.00	61.00 (59.00-64.00)
T022	Both	3.43 (0.38)	2.70-4.20	3.45 (3.30-3.60)	56.55 (18.76)	3.00-100.00	54.50 (50.50-60.00)	62.75 (7.28)	54.00-82.00	61.00 (58.50-64.00)
VO-29	Females	3.74 (0.14)	3.60-4.10	3.70	56.60 (12.37)	32.00-79.00	56.00	70.00	51.00-109.00	65.00

				(3.70– 3.80)			(52.00– 63.00)	(16.10)		(62.00– 75.00)
VO-29	Males	3.48 (0.18)	3.20–3.70	3.50 (3.40– 3.60)	64.60 (6.92)	51.00–76.00	64.00 (62.00– 70.00)	67.00 (8.26)	60.00–88.00	64.00 (63.00– 70.00)
VO-29	Both	3.61 (0.21)	3.20–4.10	3.65 (3.50– 3.70)	60.60 (10.58)	32.00–79.00	62.00 (54.50– 67.50)	68.50 (12.55)	51.00– 109.00	64.00 (62.50– 71.50)

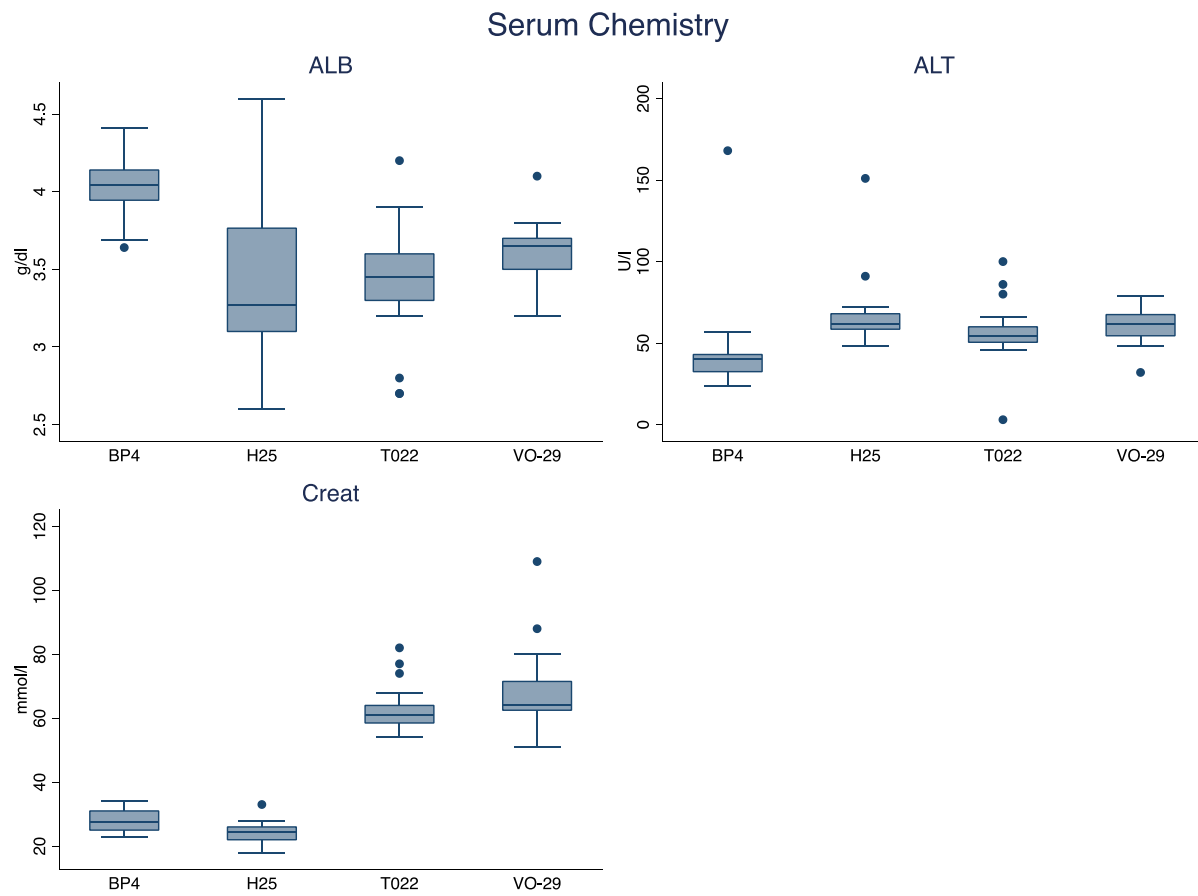


Figure 4.3: Panel of box plots for serum chemistry variables ALB, ALT and Creat.

Key: ALB; Albumen, ALT; Alanine transaminase, **Creat**; Creatinine

4.2 Meta-analysis

4.2.1 White blood cells

There were instances of evidence of heterogeneity (p-values <0.05) across subgroups and overall. However, for most of the parameters there was no evidence of heterogeneity in one subgroup/gender and overall. For instance, there was no heterogeneity among female rats ($I^2 = 36.3\%$, $p - value = 0.195$) but was observed among males ($I^2 = 81.2\%$, $p - value < 0.001$) and both genders combined ($I^2 = 67.3\%$, $p - value = 0.027$) in the forest plot on absolute monocytes. In general, majority of mean values of the measured WBC pathological variables fell within the range of the Charles River reference intervals provided in table 3.2 above.

Male SD rats had a pooled mean WCC of $7.33 \times 10^9/l$ which fell within range of the Charles Rivers intervals of $1.98-11.06 \times 10^9/l$ while female rats pooled mean WCC ($8.64 \times 10^9/l$) fell outside the Charles Rivers range of $0.96-7.88 \times 10^9/l$. In addition, the overall mean for both male and female rats of $8.03 \times 10^9/l$ fell within range of the Charles Rivers intervals (CRI). Heterogeneity was observed on male SD rats only (p-value=0.008) and overall for both genders (p-value=0.015) but was marginally significant for female (p-value=0.049) SD rats (Figure 4.4).

The pooled absolute lymphocytes mean counts fell within range of the CRI and were estimated at 5.98 (CRI: $0.68-6.8 \times 10^9/l$), 6.69 (CRI: $1.19-9.45 \times 10^9/l$) and $6.34 \times 10^9/l$ for females, males and overall respectively. In addition, statistical heterogeneity was observed across the four studies when stratified by gender and overall (p-values<0.05). Meta-analysis results on lymphocytes ($\times 10^9/l$) are presented in figure 4.5 below.

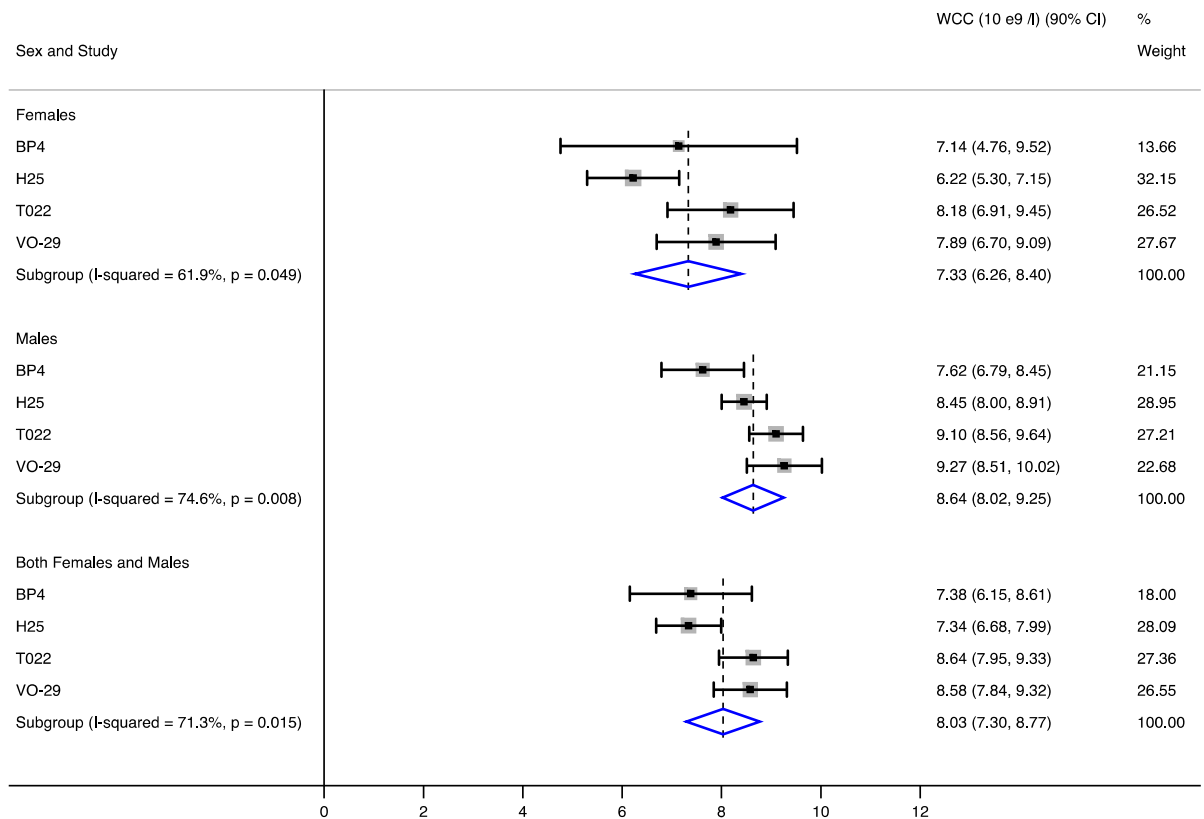


Figure 4.4: Forest plot for white blood cells counts. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

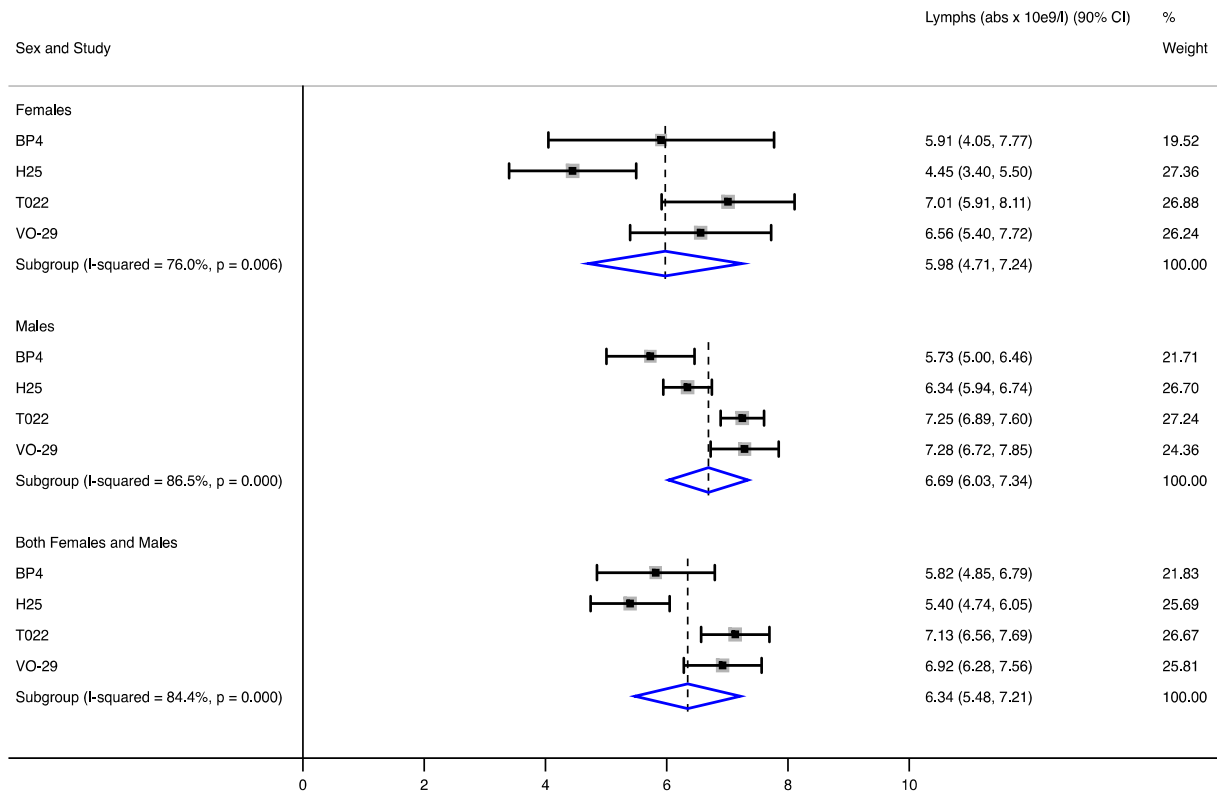


Figure 4.5: Forest plot for absolute lymphocytes. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled lymphocytes (%) mean values fell within range of the CRI and were estimated at 83.7% (CRI: 48.9-88.1%), 77.79% (CRI: 44.7-87.1%) and 80.31% for females, males and overall, respectively. There was **no** statistical significance on heterogeneity across the four studies when stratified for both females (p-value=0.064) and males (p-value=0.080) but on the overall estimates statistical heterogeneity was observed (p-value=0.027). Meta-analysis results on lymphocytes (%) are presented in figure 4.6 below.

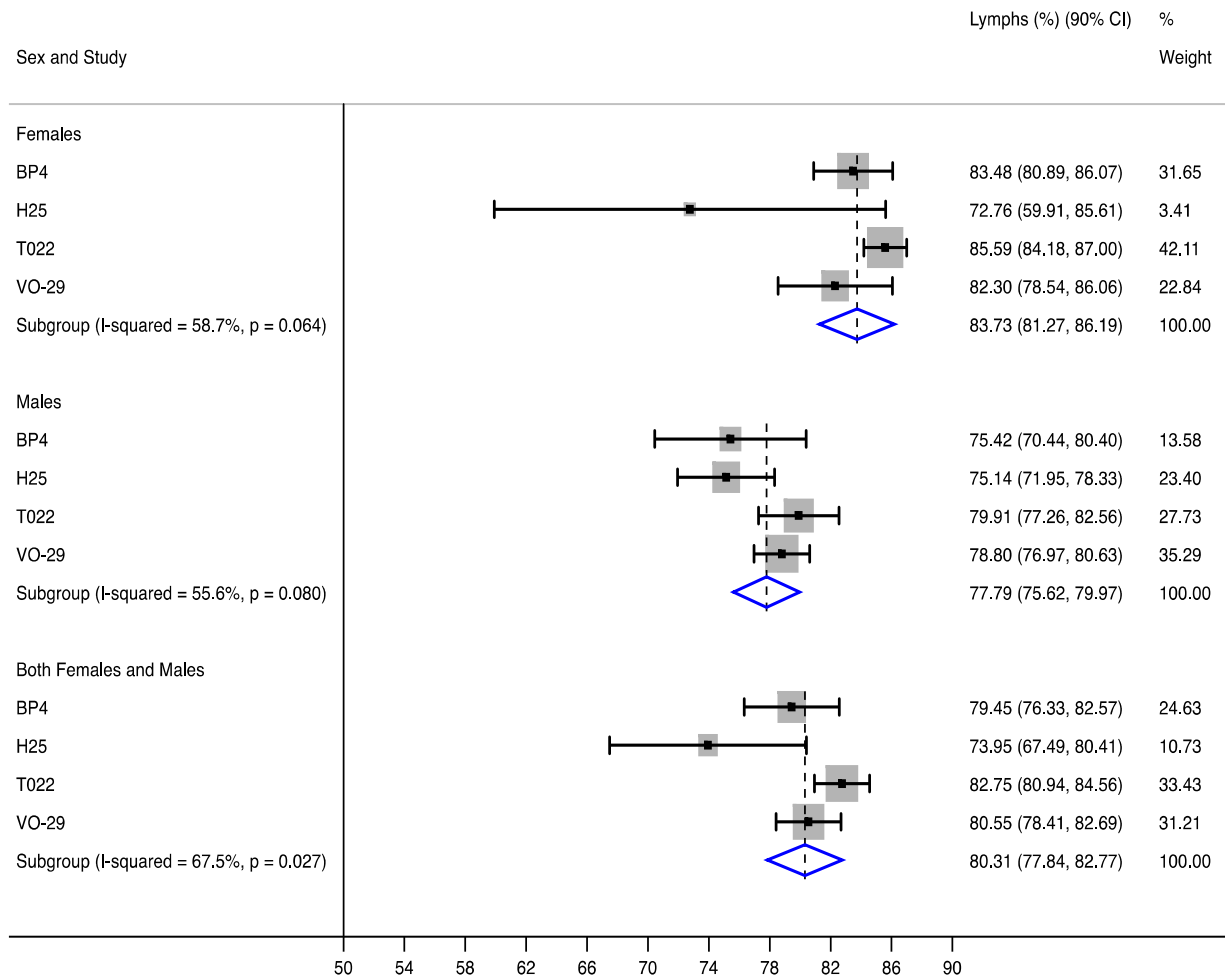


Figure 4.6: Forest plot for lymphocytes (%). The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and 90% the collective confidence interval for all groups are presented under subgroup.

The pooled platelets mean counts were estimated at $646.1 \times 10^9/l$, $693.7 \times 10^9/l$ and $671.6 \times 10^9/l$ for females, males and both respectively. These estimated pooled values fell within range of the CRI provided in table 3.2 above at $599-1144 \times 10^9/l$ and $574-1523 \times 10^9/l$ for female and male SD rats respectively. There was evidence of statistical heterogeneity across the four studies among female rats (p-value=0.03), on male rats (p-values<0.001) and on both males and females (p-value<0.001) as shown in figure 4.6 below.

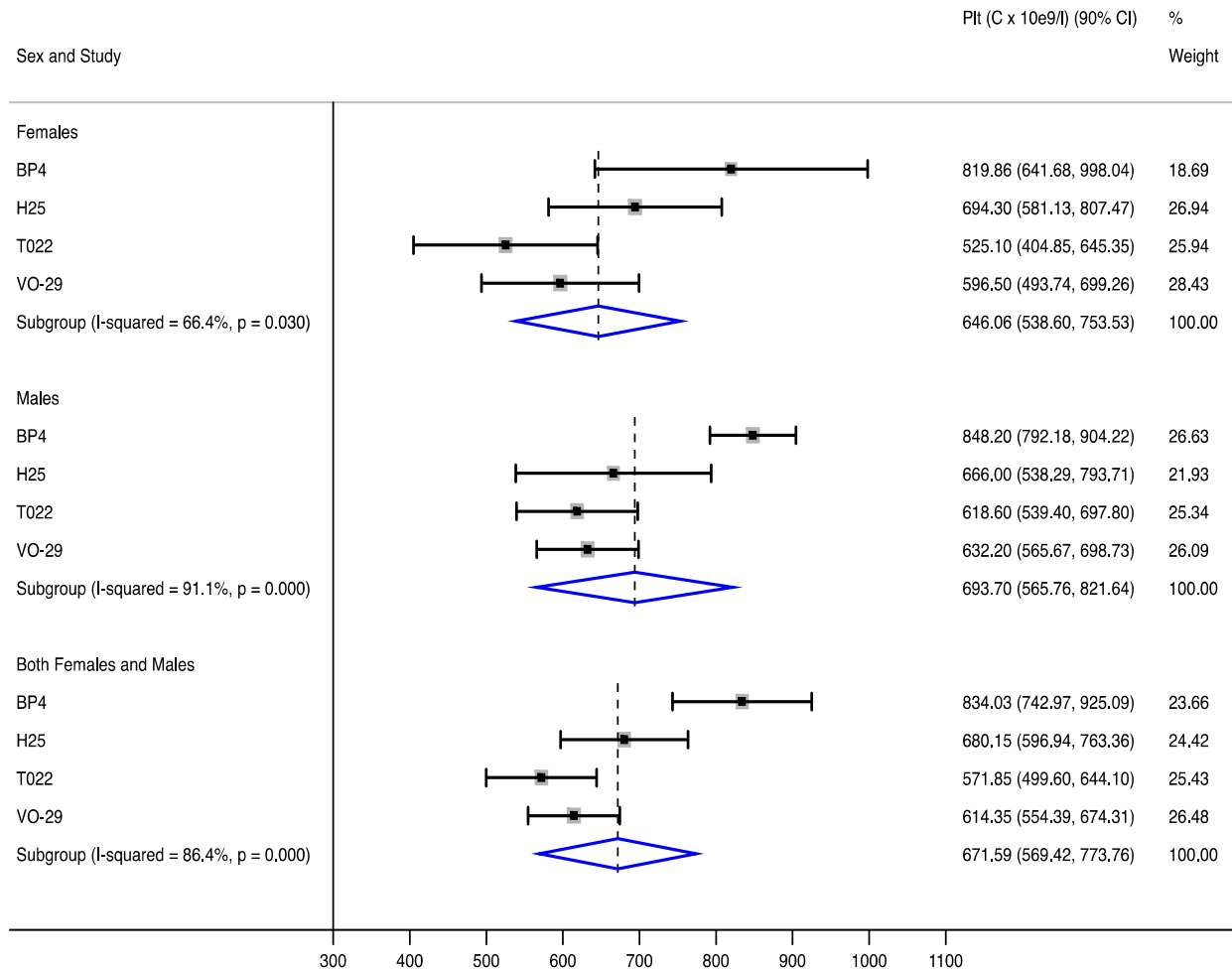


Figure 4.6: Forest plot for platelets counts. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The monocytes (%) CRI for both males and females were 1-3.6% (Table 3.2) and it was observed that female SD rats fell within range (3.07%) while male rats slightly fell above the range at 3.85% (Figure 4.7). Statistical heterogeneity was not observed across gender and overall (p-values>0.05).

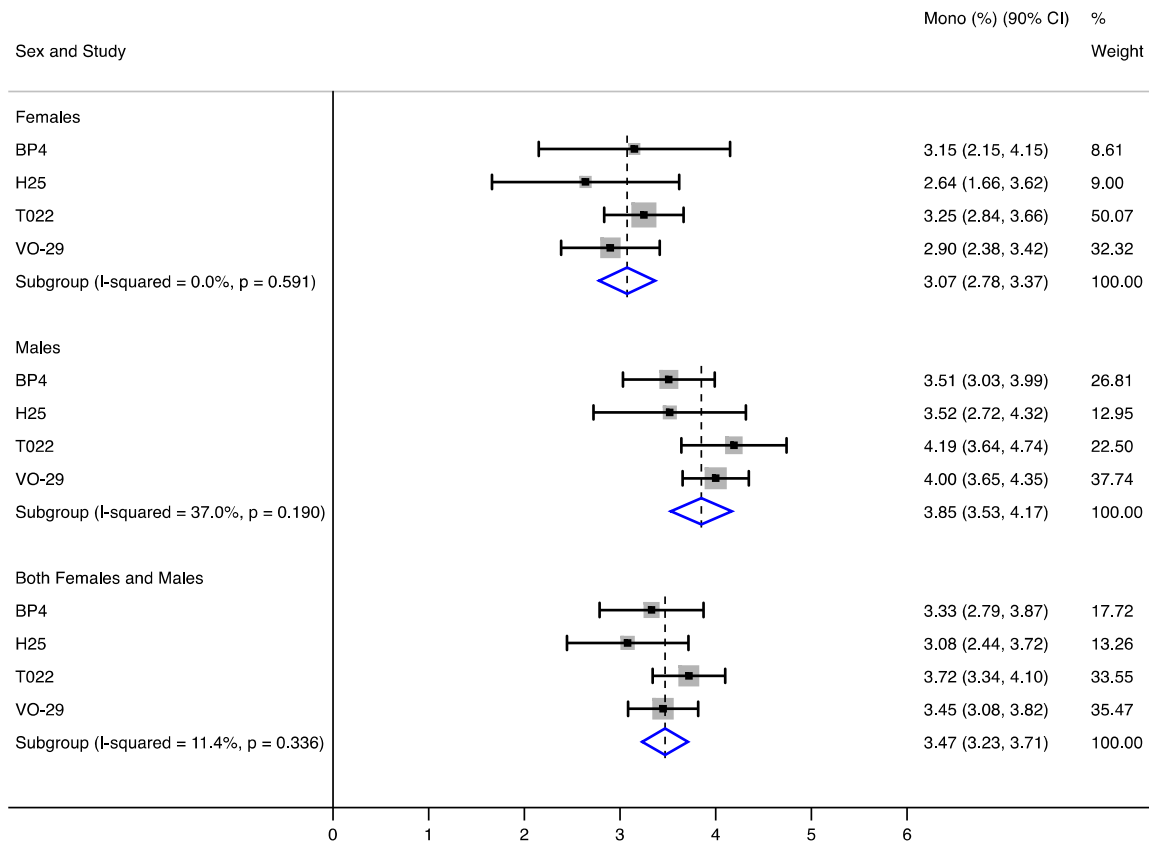


Figure 4.7: Forest plot for monocytes (%). The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

Similarly, the pooled absolute monocytes counts $\times 10^9/L$ was higher than the CRIs for female SD rats at 0.23 (CRI: 0.01-0.13), male rats at 0.33 (CRI: 0.03-0.27) and overall (0.28) as shown in figure 4.8. Statistical heterogeneity was only observed for male SD rats (p-value=0.001) and overall (p-value=0.027). The CRI ranges reported in table 3.2 for EoS (%) 0.4-4% for females and 0.3-4.7% for males. From the meta-analysis, female SD rats had Eosinophiles (EoS) (%) of 1.28%, males 1.27% and both had 1.25% which fell within CRI ranges. However, there was lack of statistical heterogeneity across studies was only observed for males only (p-value=0.064) as shown in figure 4.9.

The pooled neutrophils (%) mean values fell within range of the CRI and were estimated at 15.2% (CRI: 8.8-43.8%), 17.94% (CRI: 9-49.3%) and 16.60% for females, males and overall, respectively. There was evidence of statistical heterogeneity across the four studies when stratified among male SD rats (p-value=0.042) and **none** for both genders (p-value=0.056). Meta-analysis results on lymphocytes (%) are presented in figure 4.10 below.

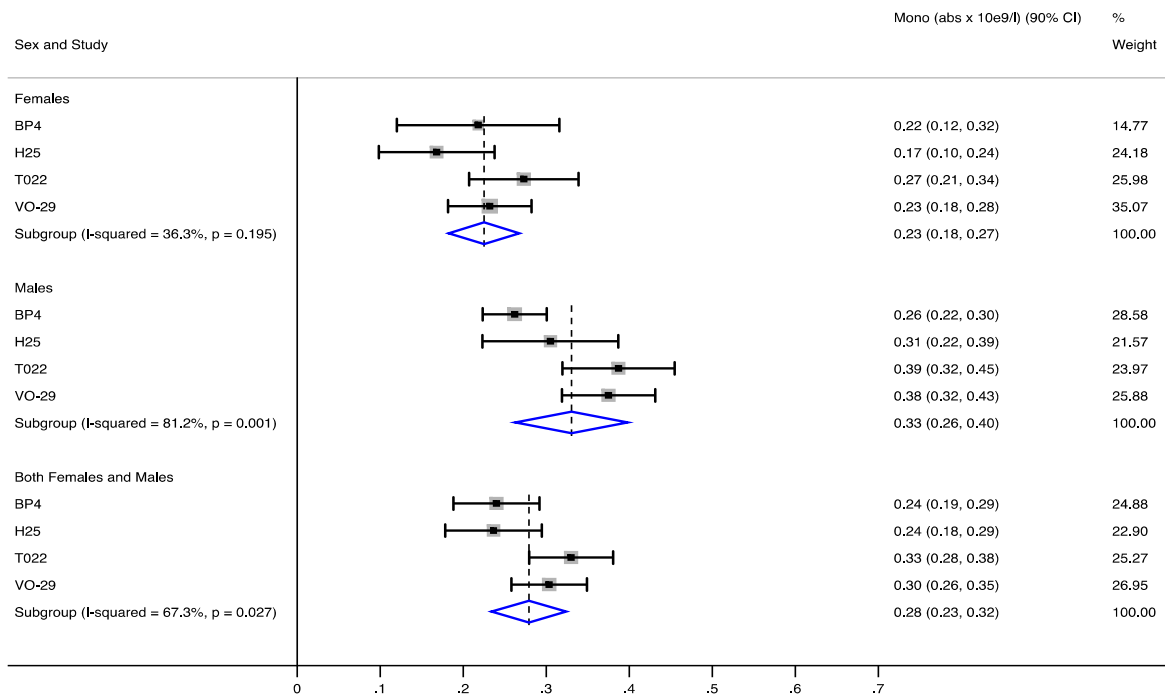


Figure 4.8: Forest plot for absolute monocytes. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

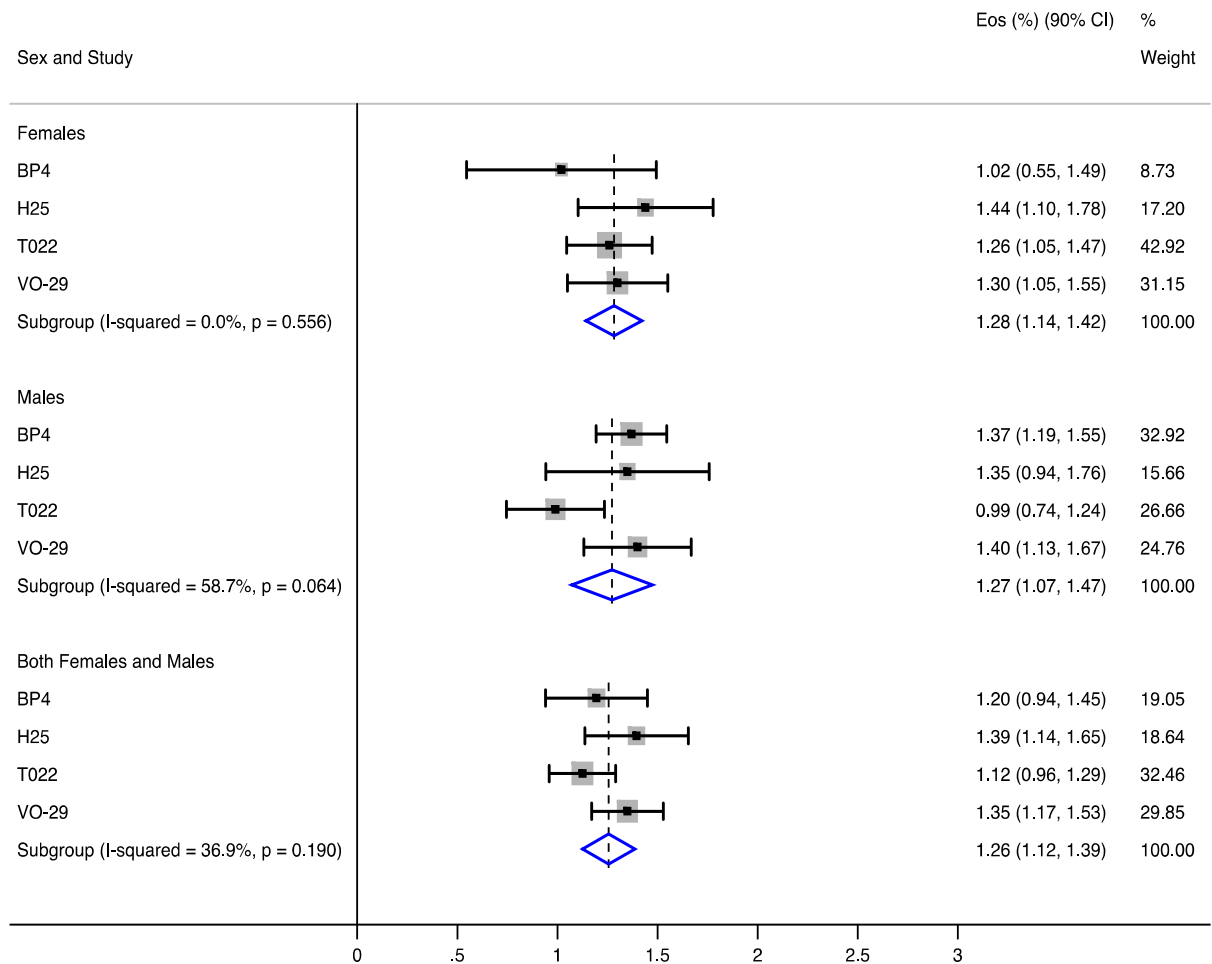


Figure 4.9: Forest plot of Eosinophils (%). The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

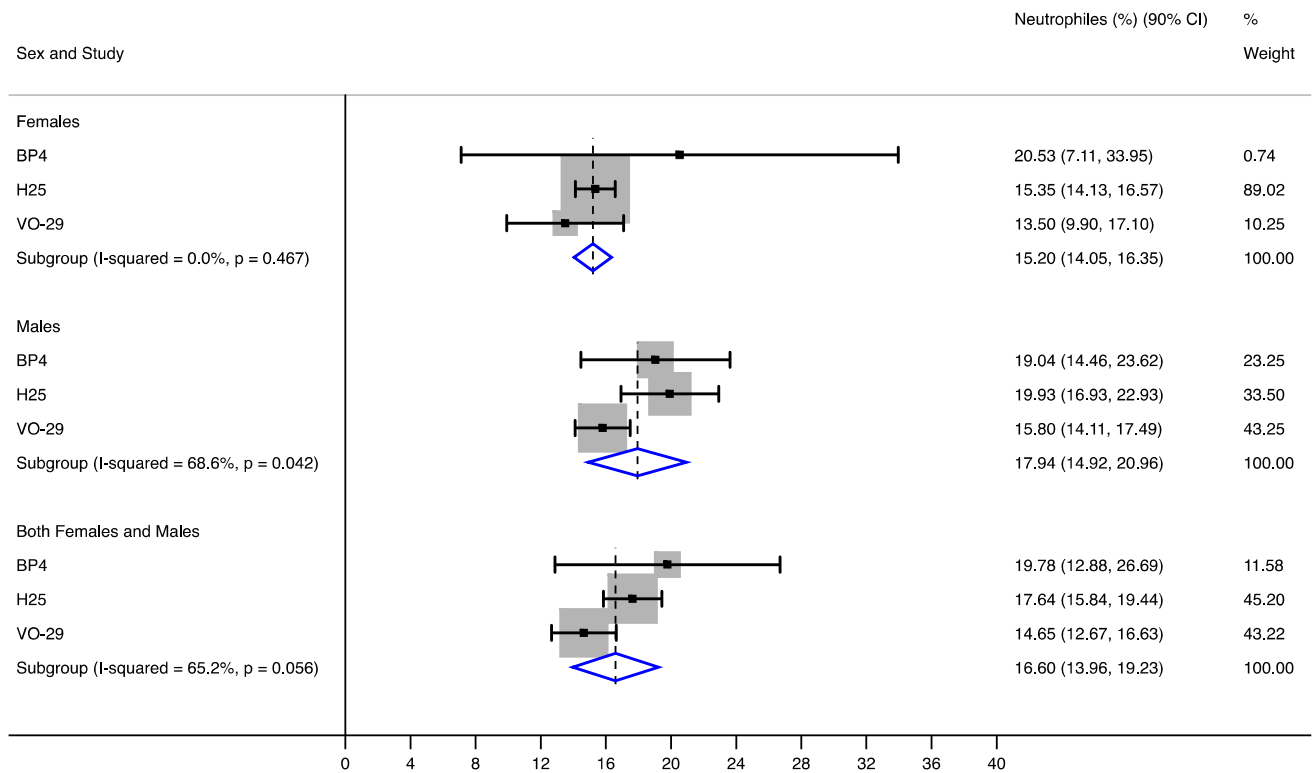


Figure 4.10: Forest plot of Neutrophils (%). The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled absolute neutrophil counts for females was 1.00 (CRI: 0.15 - 1.11) $\times 10^9/L$ and for male SD rats was 1.54 (CRI: 9 - 49.3) $\times 10^9/l$ and an overall mean of 1.29 . Male SD rats fell below the CRI but female SD rats had absolute neutrophile counts that fell within range (Figure 4.11). Statistical heterogeneity was not observed across gender and overall (p -values >0.05).

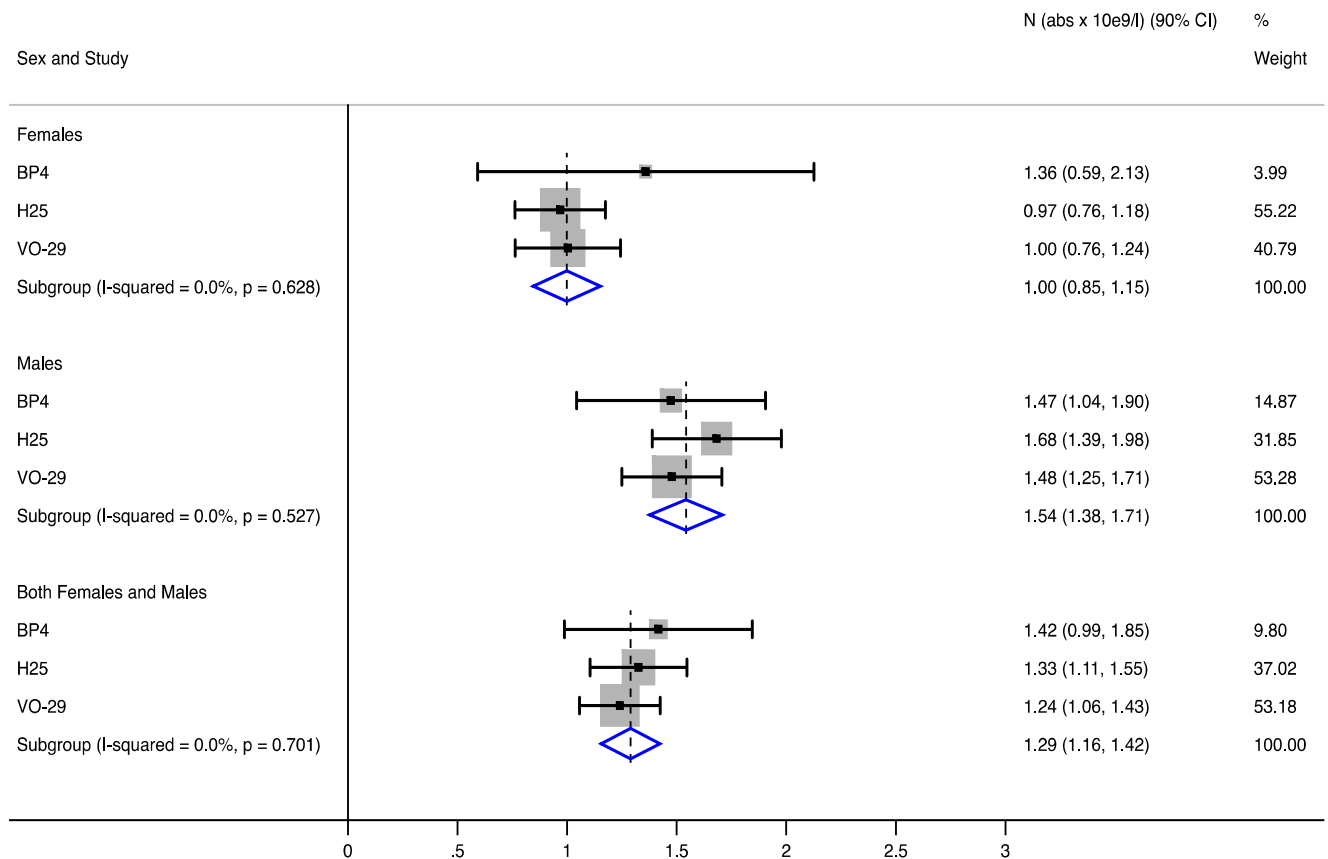


Figure 4.11: Forest plot of absolute Neutrophils. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

4.2.2 Red Blood Cells

Among the seven red blood cell variables under investigation, evidence of statistical heterogeneity was observed for nearly all parameters (p -value <0.05) except for Hb among females (p -value=0.192) and no significance for both genders combined (p -value=0.094). In addition, wide confidence intervals were observed on mean corpuscular haemoglobin (MCH) (females and overall) and haematocrit (Ht) (males and overall). A comparison of the calculated mean parameter values showed that most of the red blood cell (RBC) measurements values were within the range of the Charles River reference intervals.

The pooled mean corpuscular volume (MCV) value (figure 4.12) among female SD rats was 52.26 (CRI: 50.3-57), male rats 49.66 (CRI: 46.3-56.2) and both (50.90%). The pooled values fell within range of the CRIs and statistical heterogeneity was observed for across each gender separately and combined for both genders (p -values <0.05).

The pooled MCH (g/dl) mean values fell below the range of the CRI and were estimated at 9.89 (CRI: 17.6-20.3), 9.24 (CRI:16.3-19.5) and 9.56 for females, males and overall, respectively. There was statistical heterogeneity across the four studies when stratified by gender (p -values <0.001). Meta-analysis results on MCH (g/dl) are presented in figure 4.13 below.

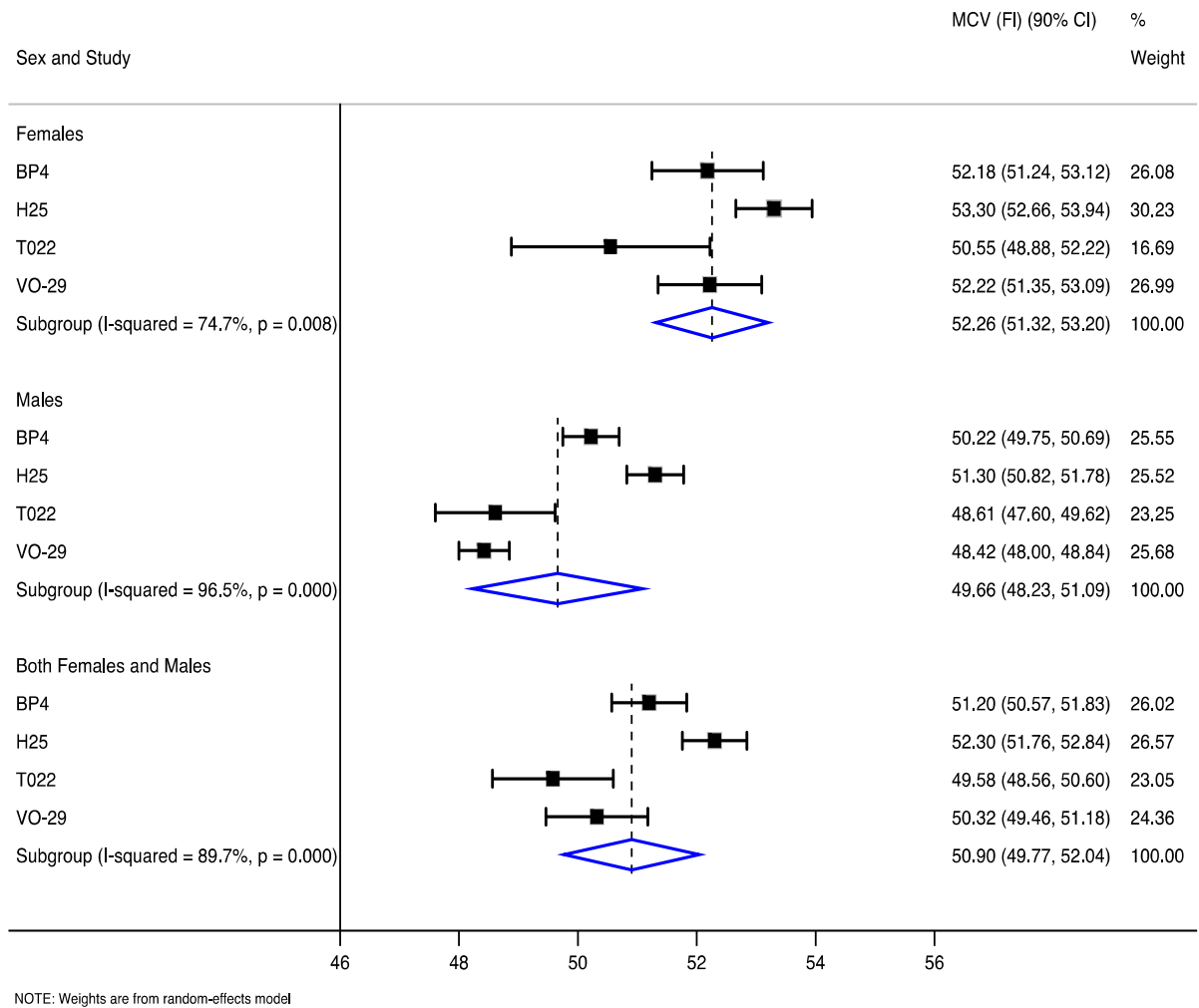


Figure 4.12: Forest plot for MCV. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

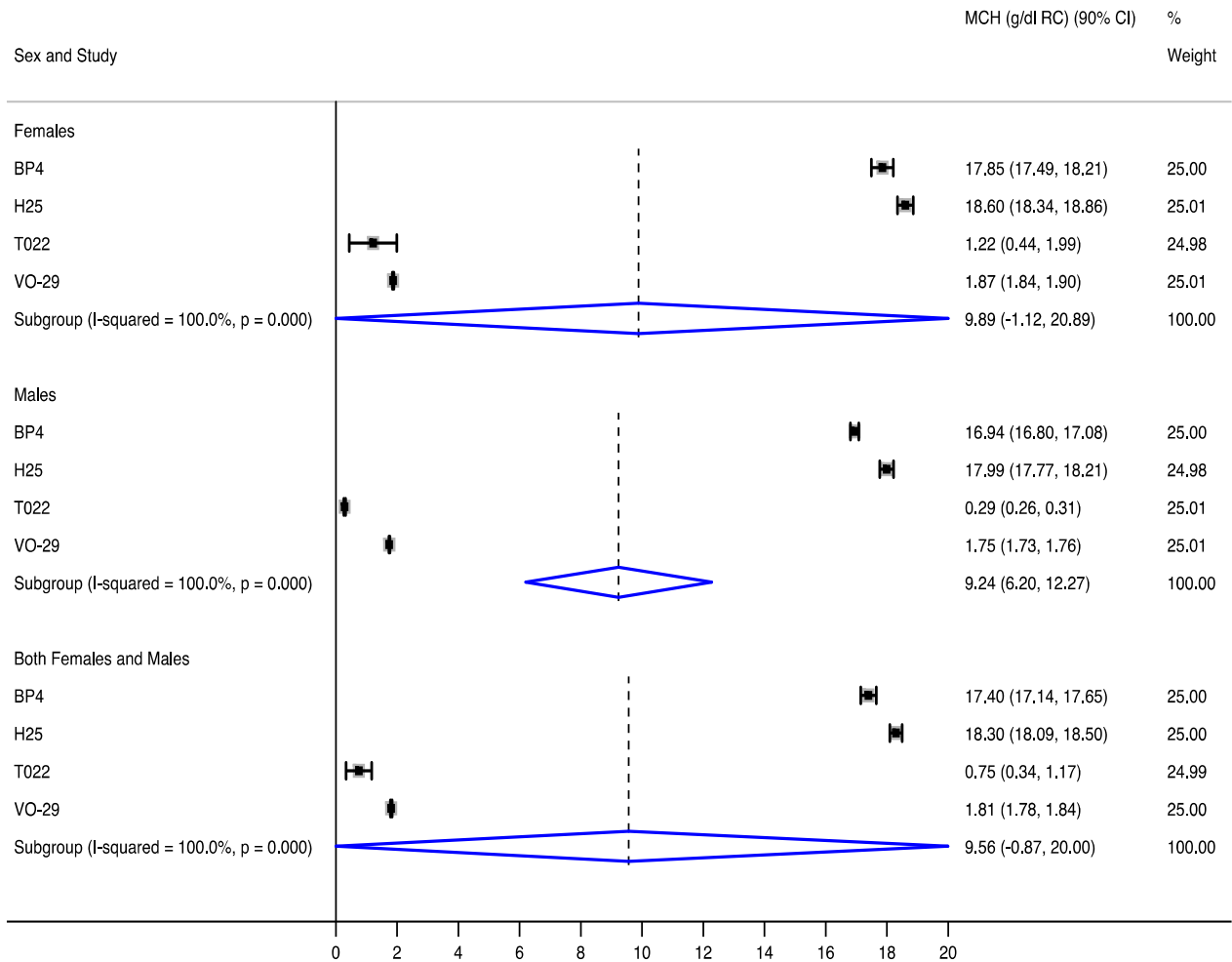


Figure 4.13: Forest plot for MCH. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The mean MCHC values (g/dl) were 34.93 (CRI: 32.2-37.8), 35.29(CRI: 31.9-38.5) and 35.05 for females, males and both respectively. All the pooled values were within range of the Charles Rivers intervals. In addition, there was statistical heterogeneity across each gender and overall (p-values<0.001). These results are presented in figure 4.14 below. With the calculated reference interval starting from 0, we have to conclude that there was fault with the analysis as it is physiologically impossible to have a value of 0.

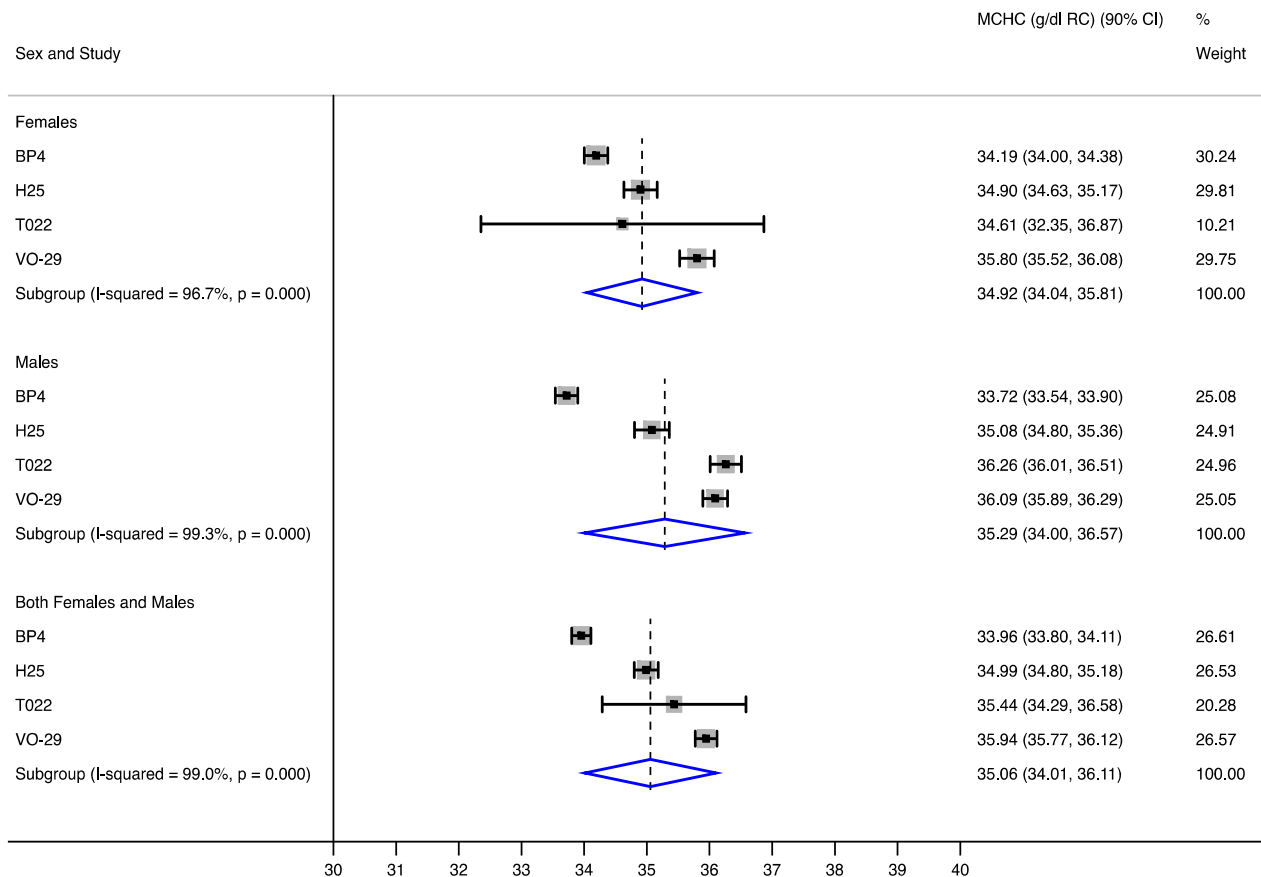


Figure 4.14: Forest plot for MCHC. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled RWD value (figure 4.15) among female SD rats was 14.19% (CRI: 10.6-14.6), male rats 14.86 (CRI: 11.6-16.2) and both (15.01%). The pooled values fell within range of the CRIs and statistical heterogeneity was observed for across each gender and overall for both genders (p-values<0.001).

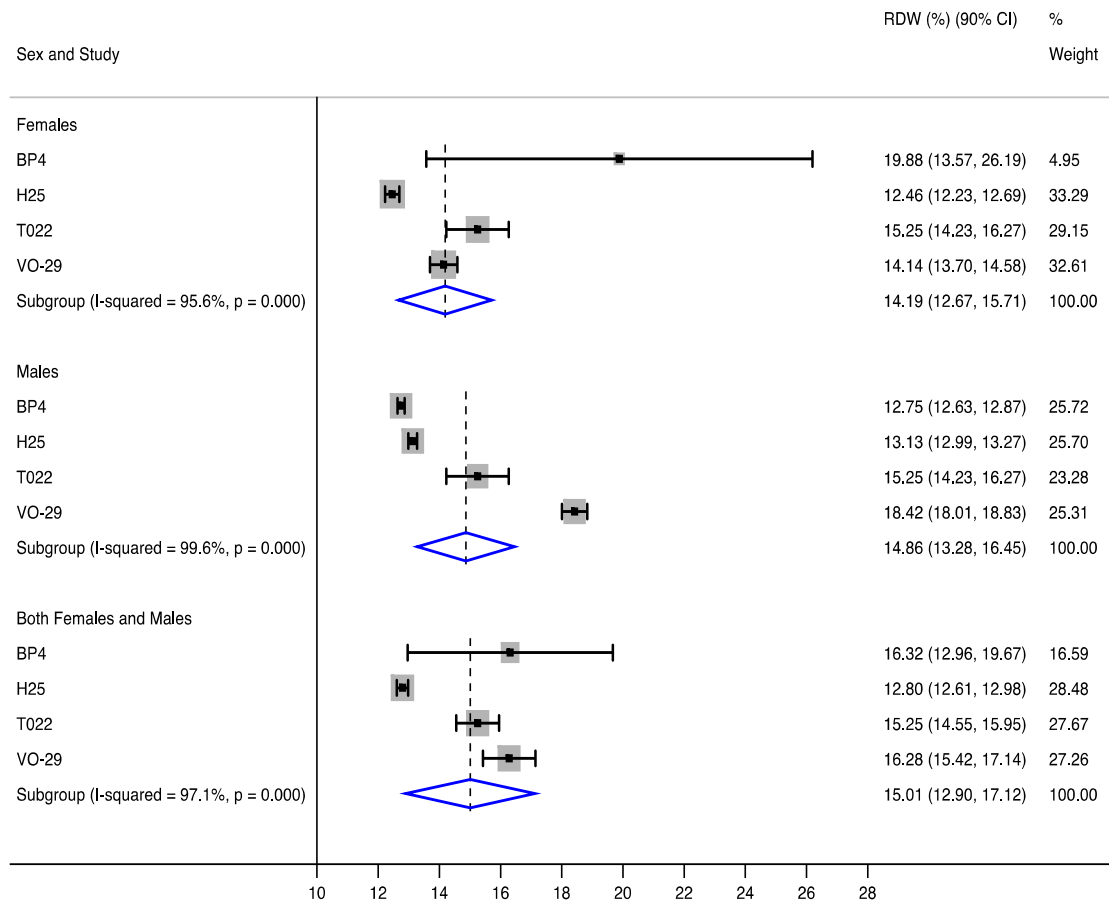


Figure 4.15: Forest plot for RDW. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The overall mean RCC value ($\times 10^{12}/l$) was 9.09 for both female and male SD rats. When the analysis was stratified by gender, female rats had pooled mean of 8.17 (7.74-9.24) while male rats had a mean of 11.53 (7.62-9.99). RCC values for female rats were within range of the respective CRI while male RCC values were slightly elevated above the upper bound of the respective CRI. Moreover, statistical heterogeneity was observed across the four studies for each gender separately and both genders put together (figure 4.16).

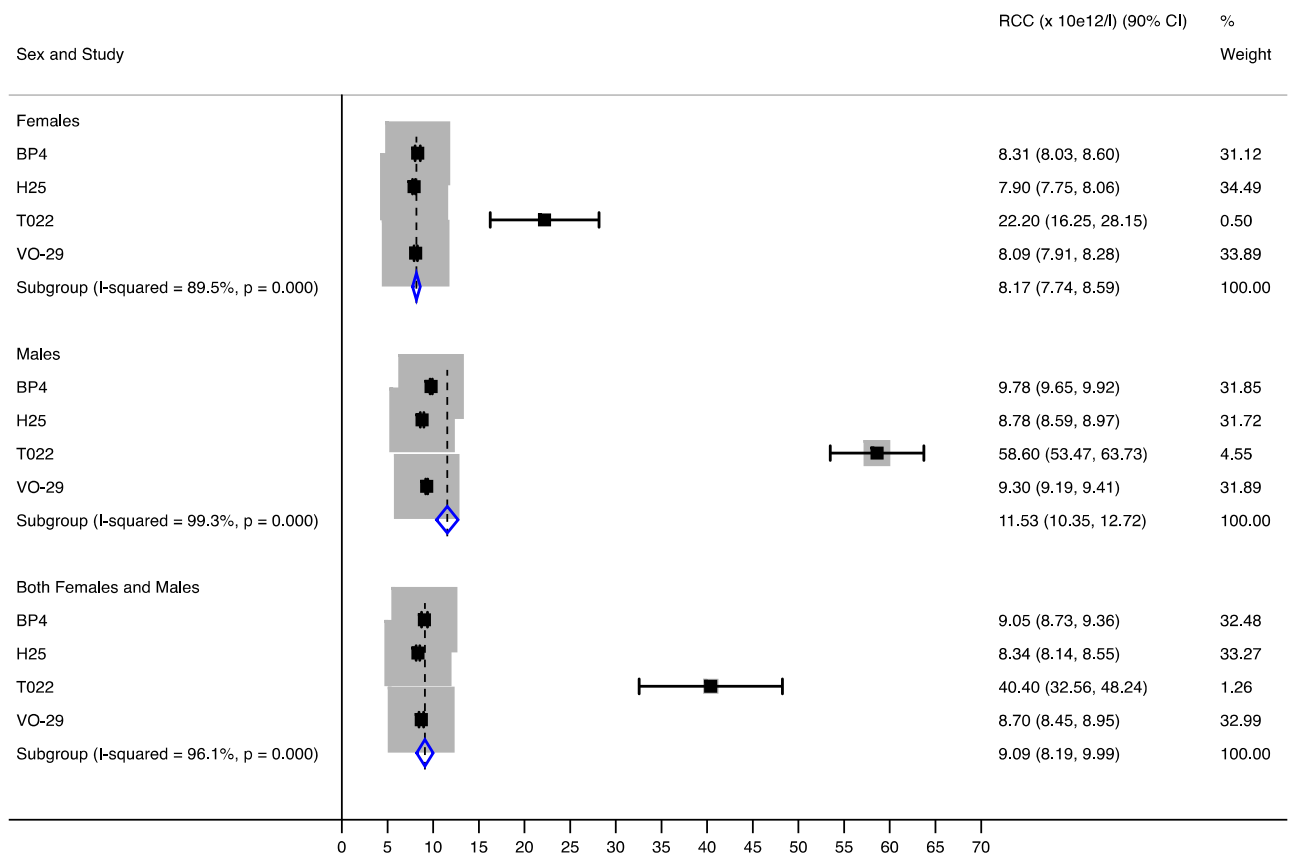


Figure 4.16: Forest plot for RCC. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled mean haemoglobin (Hb) values across the four studies were 14.83 g/l, 16.25 g/l and 15.51 g/l for females, males and both genders. These values were within the CRI ranges for the respective genders of (13.7-17.2 g/l) for females and (13.6-17.4 g/l) for males. Statistical heterogeneity across the four studies was observed among male SD rats (p-value<0.001) but not among female SD rats (p-value=0.192) and marginally for both genders (p-value=0.094) as shown in figure 4.17.

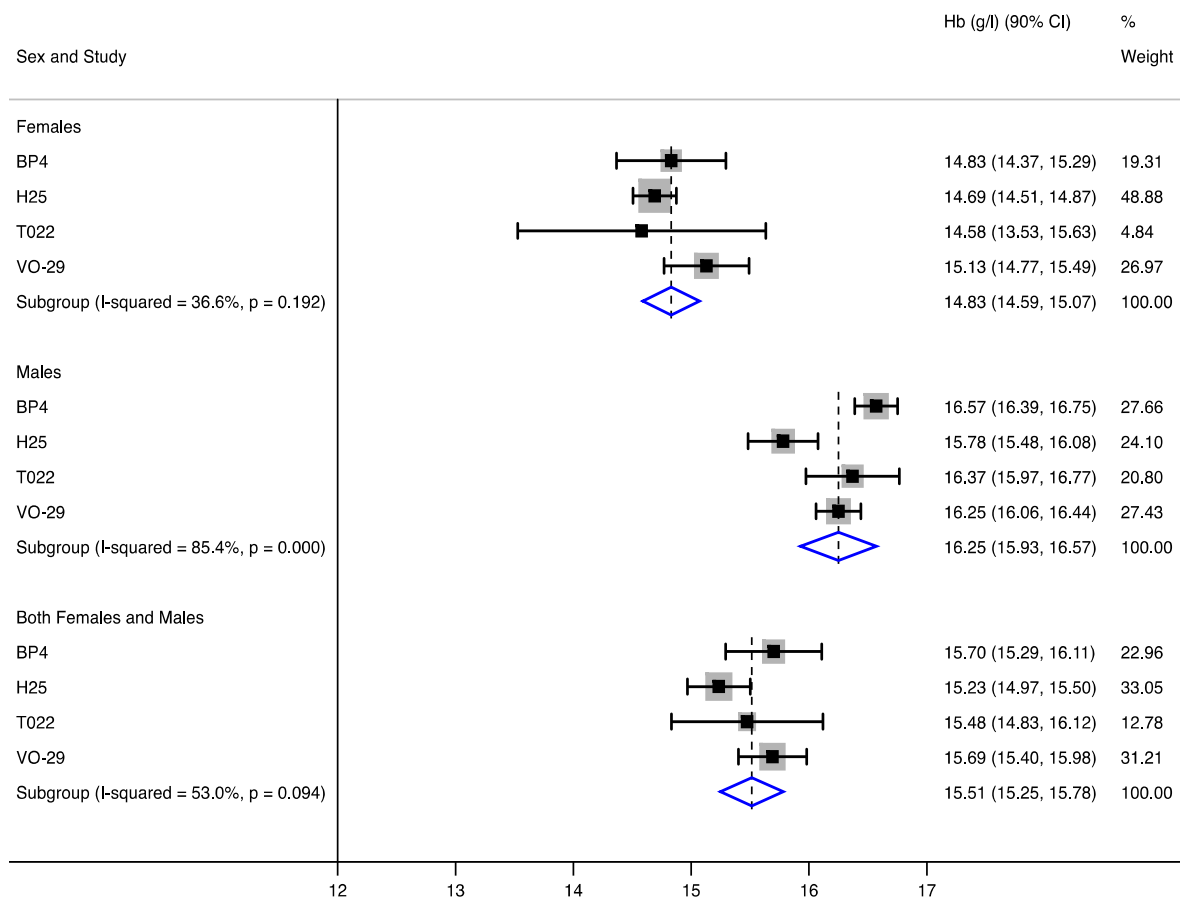


Figure 4.17: Forest plot for HB. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and 90% confidence interval for all groups are presented under subgroup.

The pooled Ht (l/l) mean values fell below the range of the CRI and were estimated at 17.90 (CRI: 38.5-49.20), 28.38 (CRI: 38.5-52) and 24.86 for females, males and overall, respectively. There

was statistical heterogeneity across the four studies when stratified by gender and both genders (p -values < 0.001). Meta-analysis results on Ht (l/l) are presented in figure 4.18 below. Since the value had a range from 0, it is likely that the value obtained in the meta-analysis is not useable as it is not physiologically possible to have a Ht value of 0.

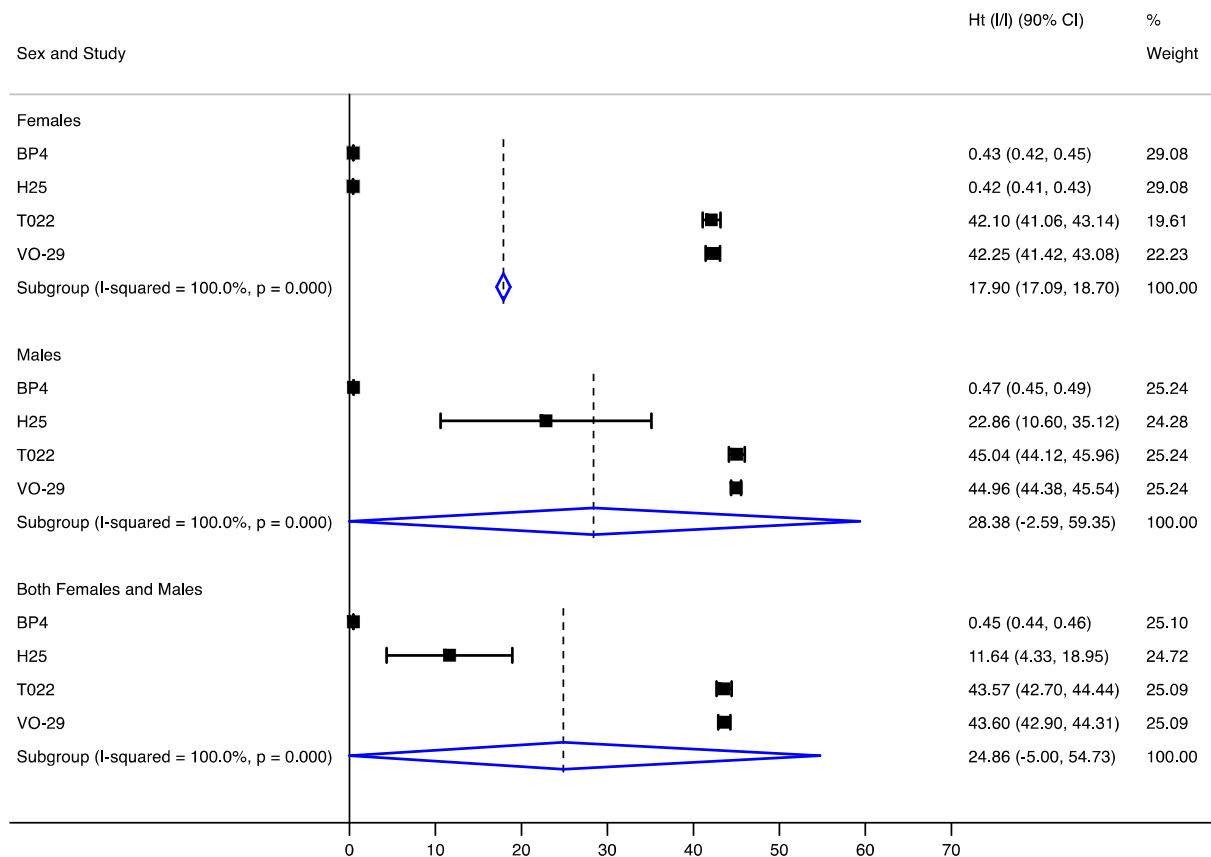


Figure 4.18: Forest plot for Ht. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

4.2.3 Serum chemistry

Male SD rats had a pooled mean ALB of 3.73 g/dl which fell within range of the Charles Rivers intervals of 3.6-4.7 g/dl while female rats had a pooled mean ALB value of 3.54 g/dl which slightly fell below the Charles Rivers range of 3.7-5.8 g/dl. In addition, the overall mean for both male and female rats of 3.64 fell within range of the Charles Rivers intervals (CRI). Statistical heterogeneity was observed across the four studies on both genders separately and combined (p -values <0.001) (Figure 4.19).

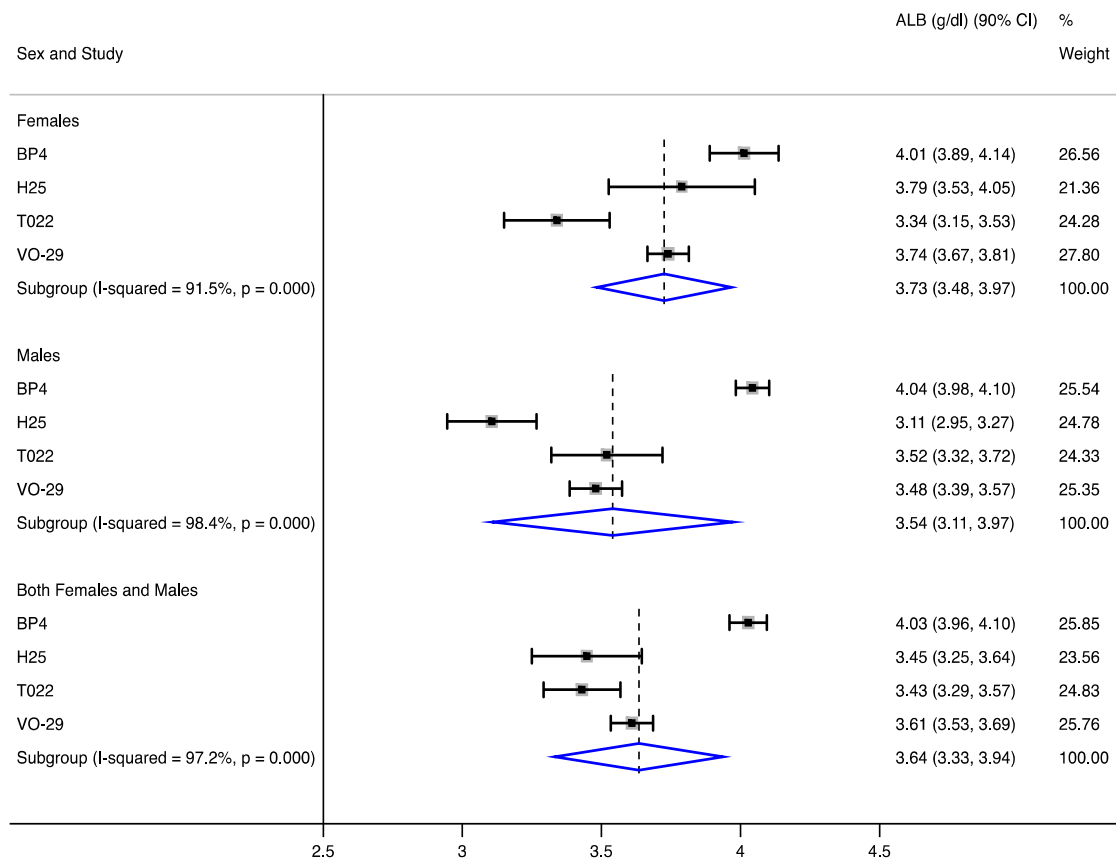


Figure 4.19: Forest plot for ALB. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled ALT (U/l) mean values fell within range of the CRI and were estimated at 51.57 U/l (CRI: 14-64 U/l), 64.25 (CRI: 19-48 U/l) and 58.00 U/l for females, males and overall, respectively. There was statistical heterogeneity across the four studies for females (p-value <0.001) and both genders (p-value=0.007). However, heterogeneity was not observed among male SD rats (p-value=0.588) as shown in figure 4.20 below.

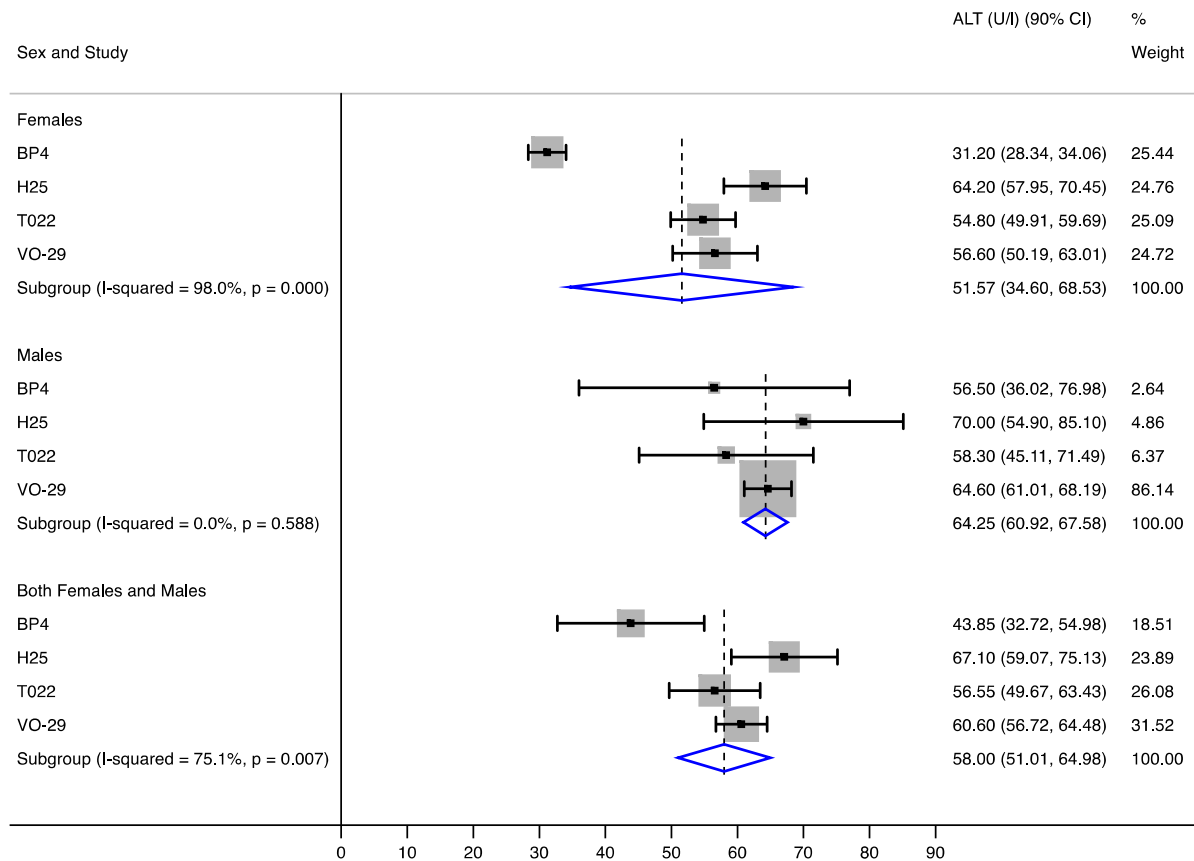


Figure 4.20: Forest plot for ALT. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

The pooled creatinine (mmol/l) mean values fell within range of the CRI and were estimated at 46.49 and 45.69 mmol/l for females, males and overall, respectively. There was statistical heterogeneity across the four studies when stratified by gender and both genders combined (p-values<0.001). Meta-analysis results on creatinine (mmol/l) are presented in figure 4.21 below.

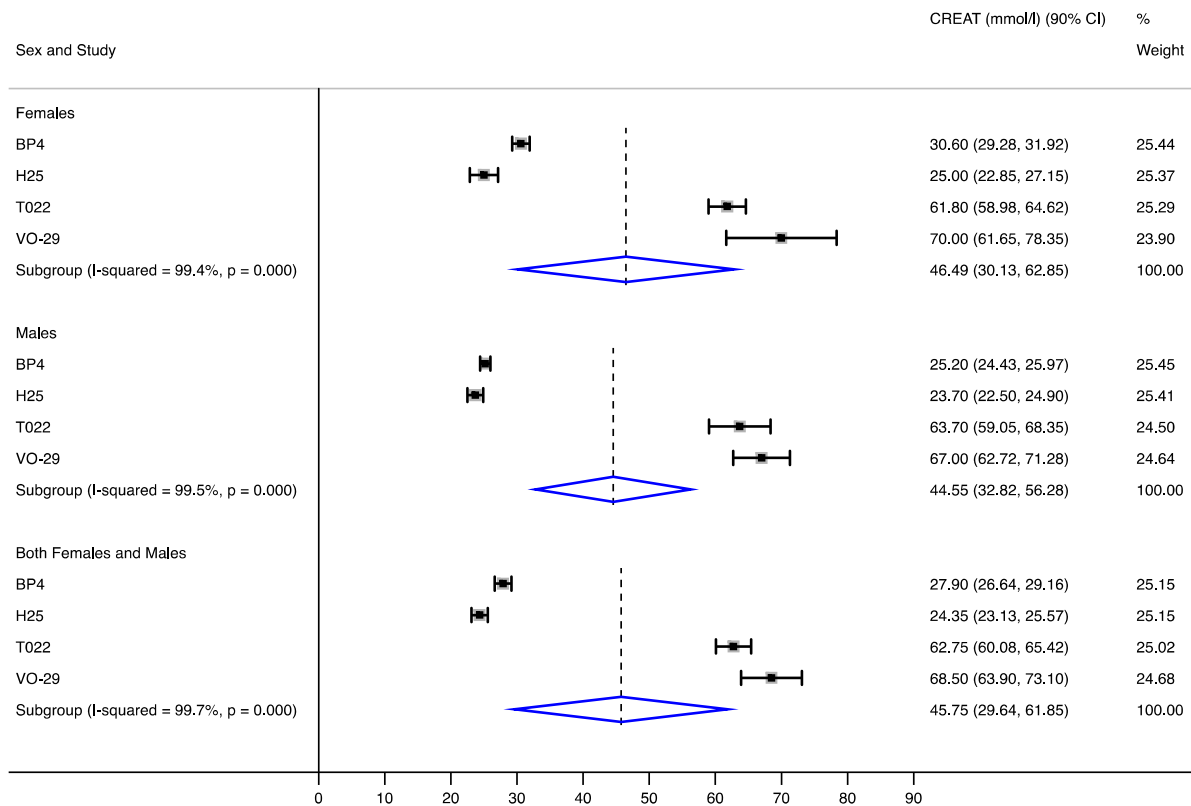


Figure 4.21: Forest plot for Creatinine. The figure shows the values for each parameter independent and dependent on sex. The blue diamond shows the results from the meta-analysis for the particular sub-group. Also presented are the individual study means and 90% confidence intervals. From the indicated weighting, determined from the random effects model, the pooled means and the collective 90% confidence interval for all groups are presented under subgroup.

4.2.4 Meta-analysis Summary

A summary of the new reference intervals obtained from the meta-analysis results in comparison to the Charles Rivers reference intervals is presented in table 4.8 below.

From the meta-analysis conducted on red blood cells parameters, it was established that the new reference intervals had a higher lower bound for Hb, RCC, MCV, MCHC, RDW and WCC. In addition, these new reference intervals had shorter ranges compared to the CRI with the exception of Ht intervals on males which was wider reference interval (0-60 l/l) compared to CRI (38.5-52 l/l). Among female SD rats, the new reference interval was 0-20.89 g/dl was wider and higher than the CRI (17.6-20.3 g/dl) for MCH. Moreover, male SD rats had a wider MCH reference interval 6.2-12.27 g/dl compared to CRI of 16.3-19.5 g/dl.

Among white blood cells parameters, narrower reference intervals were observed for all parameters. Specifically, lymphocytes (%) had a higher lower bound in the new reference intervals of 81.67-86.38% among females and 75.81 – 80.08% among males compared to CRIs of 48.9-88.1% and 44.7-87.1% respectively. Platelets had lower reference intervals compared to CRI at $535-750 \times 10^9/l$ verses $599-1144 \times 10^9/l$ and $566-823 \times 10^9/l$ verses $574-1253 \times 10^9/l$ for females and males respectively.

In the serum chemistry parameters, ALB the new reference intervals were 3.48-3.97 g/dl among female SD rats and 3.11-3.98 g/dl among males and were narrower than CRIs of 3.7-5.8 and 3.6-4.7 g/dl of females and males respectively. ALT values had new reference intervals of 34.53-68.49 U/l for females and 60.27 – 68.23 U/l for males which were higher and narrower than CRIs of 14-64 U/l and 19-48 U/l for females and males respectively.

Table 4.8: Summary of reference intervals obtained by meta-analysis compared to the Charles Rivers Intervals.

Parameter	Abbreviation	Charles Rivers Intervals		New reference intervals from Meta-analysis	
		Females	Males	Females	Males
RBC	Hb	13.7-17.2	13.6-17.4	14.59 – 15.07	15.93 – 16.57
	RCC	7.16-9.24	7.62-9.99	7.74 – 8.59	10.35 – 12.72
	Ht	38.5-49.20	38.5-52	17.90 – 18.70	0-59.35
	MCV	50.3-57	46.3-56.2	51.32 – 53.20	48.23 – 51.10
	MCH	17.6-20.3	16.3-19.5	0 - 20.89	6.2-12.27
	MCHC	32.2-37.80	31.9-38.5	34.04 – 35.81	34.0-36.57
WBC	RDW	10.6-14.6	11.6-16.2	12.67– 15.71	13.28 – 16.45
	WCC	0.96-7.88	1.98-11.06	6.26 – 8.40	8.02 – 9.25
	Neutrophiles (%)	8.8-43.8	9-49.3	14.05 – 16.35	14.92 – 20.96
	Lymph (%)	48.9-88.1	44.7-87.1	81.27 – 86.19	75.62 – 79.97
	Mono %	1-3.6	1-3.6	2.78 – 3.37	3.53 – 4.17
	Eos %	0.3-4.7	0.4-4	1.14-1.42	1.07 – 1.47
	Neutrophiles (abs)	0.15-1.11	9-49.3	0.85 – 1.15	1.38 – 1.71
	Lymph (abs)	0.68-6.8	1.19-9.45	4.71 – 7.24	6.03 – 7.34
	Mono (abs)	0.01-0.13	0.03-0.27	0.18 – 0.27	0.26 – 0.40
	Plt	599-1144	574-1253	538.6 -753.5	565.76-821.64
	ALB	3.7-5.8	3.6-4.7	3.48 – 3.97	3.11 – 3.97
	ALT	14-64	19-48	34.60 – 68.53	60.92 – 67.58
	Creat	0.3-0.60	0.3-0.5	30.13 – 62.85	32.82 – 56.28

*Highlighted cells indicate reference intervals that are not biologically possible

CHAPTER 4: DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS.

In this chapter, a discussion of the key findings from the previous chapter is presented. In addition, the strengths and limitations of the study are presented as well as the overall conclusions are presented.

4.1 Discussions

Interpretation of Toxicity Studies for Clinical Pathology Parameters

Everds (2015) states that in toxicity testing, analysis is used usually on whole blood, serum and plasma. In subsequent analysis, variables evaluated are divided into hematology, clinical chemistry and clotting cascade. Variables normally evaluated in hematology include red blood cell components such as haemoglobin (Hb), red cell count (RCC), haematocrit (Ht), mean corpuscular volume (MCV) and Red cell distribution width.

Red cell count (RCC), haemoglobin and haematocrit deficiencies are an indication of anemia which may result from various causes while other variables may be useful in detecting specific organ dysfunction such as early stage kidney damage, based on concentrations of serum creatinine and albumin. An increase in white blood cells may be an indication of infection, while its decrease could indicate bone marrow suppression. Interpretation can include hepatic measurements study that will analyze liver function tests and liver enzymes such as alkaline phosphatase (ALP), alanine amino transferase (ALT) and aspartate amino transferase (AST) (Ketsia et al., 2016). Other evaluations can include platelet deficiency, which may be an indication of blood loss due to a hemorrhage, anemia or a gross injury to blood vessels where clotting is compromised resulting in more blood loss (Everds., 2015).

While the principle behind abnormalities is well accepted, it is not easy to pick up problems, as the normal for the particular individual is usually not known. Also, since every animal's pathological profile does differ slightly between individuals, standardized methods have been developed to allow for the interpretation of cases when abnormalities are present, as deviation of these variables from their homeostatic normal ranges may result in pathology. As an example, an elevated liver function enzymes count signifies severe hepatic damage, while a low protein diet and conditions such as hepato-splanchnic hypoxia, resulting in hepatic cellular damage can reduce the amount of liver enzymes (Agrawal et al., 2013)

More importantly in many cases, changes in clinical pathology variables can be an earlier indicator of organ dysfunction or injury, than overt organ failure. Gary et al., 2008 denotes that reference intervals are normally established to help clinicians interpret laboratory test results. For this, a non-parametric method is used, so that the data distribution does not matter but rather that the values are put in order of the lowest to the next lowest, and the defined range then becomes the reference interval. Meanwhile end points of the confidence limits of the interval can actually be derived from the data points. It is a common practice to use 120 data points so to achieve the 95 % central distribution of the population.

Gary et al., 2008 further states that it can be noted that given 120 observations, rank 3 is the 2.5th percentile, rank 1 and 7 defining 90 % confidence interval, while rank 118 is the 97.5th percentile. Other ranks 114 and 120 are defining the 90% confidence interval of the 97.5th percentile and partitioning normally is done for gender and age.

Through a well-accepted history of use, these reference intervals now allow for a more sensitive indicator of changes, even before histological evidence of organ damage is present. It is also well accepted that with more severe changes in these variables, they often correlate with structural changes due to their ability to exist as markers to a certain pathology, that can be diagnosed by a light microscopy (Everds, 2015; Mahl et al., 2000).

Not surprisingly, the achievement of valid results in a toxicity study occurs when there is correlation of data in a group and or individual study animals, and when these correlations lead to a clear mechanism of an underlying problem, this being an interpretation of data sets captured as raw data during the study. Concordance occurs nicely in regulated variables or when a test item has a direct clinical impact on the organs or on physiologic processes in a standardized manner. As an example, a classic example for direct impact is clinical evidence of tremors as a result of a decrease in serum calcium levels, while in a physiologic process effect, it can be evident when multifocal moderate liver necrosis exists coupled by an elevated count on ALT and AST (Everds, 2015 and Mahl et al., 2000). Regulated variables are known to maintain health, and therefore are bound to be in concordance with other variables. As expected, a result of toxicity or abnormality can be made when variables in the treated groups fall outside of the reference interval established for a particular variable.

Jones et al. (2008), indicates that unfortunately, despite the importance of reference intervals in diagnostic medicine and interpretation of study results, problems can also arise from the methods used in the development of reference intervals, with accuracy and statistical specifically being important. Influential pre-analytical factors are sampling, time of day when collecting serum, time to the onset of centrifugation, and the differences between the usage of serum as opposed to plasma in heparin. When dealing with inconsistency in results, it is important to conduct a root cause analysis which will cover a wide range of factors that can directly affect the results and their integrity, they range from measurements, materials, personnel, environment, methods, and machines (equipment). It is important to also describe the variables fully and have a standard way to use to describe them than to use words interchangeably, hence to achieve their biological variation and clinical utility (ISO 17025:2017).

It is important to note that the laboratory facilities available for testing are adequate as required to facilitate correct performance of the tests, and that laboratory environmental conditions do not invalidate the results. Attention should be provided, such as biological and chemical sterility, temperature, humidity, and radiation. It is also important to have effective separation of areas to avoid cross contamination. Equipment used should be routinely calibrated during usage, while proper maintenance must be scheduled and implemented to avoid deviation of equipment

functionality from normal specifications. Due to the sensitive nature of analytical equipment, verification of equipment should also be done as intermediate checks prior to the calibration cycle. Personnel working as analysts should be appointed based on education, qualification and experience and continual professional development. With satisfactory implementation of the above mentioned aspects, this is a preventative measure to result deviation hence protecting their integrity.

Cristina et al. (2008), states that in addition to the concerns expressed above, there can be biological reasons, as there exist the potential for the reference interval to be non-applicable to the test result as a result of genetic drift, where there is an emergence of a new strain which developed over a period of time due to genetic mutations, and the intervals falls outside the existing reference intervals.

As with other countries, South Africa researchers are involved in the preclinical evaluation of new medications, using well established models. However, unlike larger countries, there are only a limited number of breeding organizations that can supply animals. Further there is no indication, that these facilities are not able to employ breeding practices that are able to minimize the effect of genetic drift. Further there is some indication that the international available clinical pathology variables are not applicable to South African bred animals.

In this retrospective study, we attempted to evaluate the difference between parameters obtained from locally bred SD rats to the reference interval published by a reputable international breeder organization as well as to determine if local reference intervals need to be established. For this the clinical pathological variables of SD rats that formed part of healthy controls for four studies conducted at the UPBRC laboratories were used as a first step is determining if genetic drift is present, this has implications on future toxicology studies conducted on inbred SD rats which are conducted at UPBRC and possible other laboratories using SD rats in research projects. Clinical pathology variables were aggregated and analysed separately by gender and all genders combined while stratified by study. The distribution of the variables was examined using box plots and meta-analysis conducted to compare the variables across studies stratified by gender.

In this study, there were variations in the measured values across the three main categories of white blood cells, red blood cells and serum chemistry. While some variables fell within the published reference intervals, there were deviations from the normal ranges for a number of variables. For instance, absolute monocyte counts were higher than the normal ranges for both genders while mean corpuscular haemoglobin (MCH) and haematocrit (Ht) had lower values than normal ranges. This brings about an idea that Charles river reference intervals require a review to be relevant to South Africa, so as to identify any possibilities of some parameter values falling outside the normal range.

This therefore suggested that local defined reference intervals would be needed. From the data, it was established that the measured values were fairly symmetrically distributed across the studies with a few instances of outliers observed. For instance, study T022 had higher red cell count values compared to the other 3 studies and higher values of haematocrit and red cell distribution width were observed for studies T022 and V0-029. Study H25 had a wider range of ALB values compared to the remaining three studies while studies T022 and V0-29 reported the higher values of creatinine compared to studies BP4 and H25. Nonetheless the data was still analyzable by meta-analysis and resulted in the lower bounds of the new reference intervals being higher than those of the Charles Rivers intervals. This was specifically observed for haemoglobin, red cell counts, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration, red cell distribution weight and white cell count in addition to instances of wider reference intervals in other variables. Most white blood cells and serum chemistry variables had narrower reference intervals compared to the published Charles River reference intervals.

As to the exact cause of the deviations, the influencing factor(s) for variations of the measured values could not be readily established in this study. However, based on the results from other studies, we are able to speculate on a cause. With Damsch et al., 2011; de Kort et al., 2020 indicating that previous studies showing that accidental aspiration of high viscosity vehicles or by gavage-related reflux-induced lesions could also result in false changes in the control group animals, we went into the study site records and confirmed that no incidence to accidental dosing were recorded.

Also, since the animals were dosed with water as a controlled substance, toxic induced changes are not likely to occur. Finally, the post-mortem and necropsy information available for these rats, showed them to be healthy rats at the time of euthanasia, with also no indication of any inflammatory or infectious signs. As a result, we believe that the most likely cause of these differences / variations could be because of genetic drift which led to creation of altered strain of SD rats.

Festing (2010) & Gileta et al (2018), suggests that the determination of genetic drift in retrospective studies such as this one is difficult to achieve. What is important to note, was that the rats were all reported to be in-bred derived animals. While inbred strains are preferred over using outbred strains in toxicity study due to lower inter-subject variability due to lower phenotypic variability among inbred strains, their use is still dependent on strain in use being properly characterized, as genetic drift within the rat models used in toxicity studies cannot be prevented despite technological advances in the breeding process.

It is thus likely that the SD rats used in this study were not averse to any genetic drift in their South African breeding programme. As a result, it would be helpful if further genetic evaluations can be undertaken on the breeding population from which the study animals were provided, in order to ascertain how far they've deviated from their original SD breeding stock.

4.2 Strengths and limitations

This study has combined data on healthy SD rats that formed the control population of four different studies conducted at the University of Pretoria Biomedical Research Centre. This has therefore given greater evidence on the potential genetic drift among inbred SD rats used for laboratory toxicology studies. While the health of the animals under evaluation were based on clinical and necropsy findings, the presence of genetic drift will need to be confirmed with molecular techniques, the favored methods in use nowadays. Also root cause analysis would be needed as to definitively rule out other factors that may influence variation of results, including measurements, materials, personnel, environment, methods, and machines (equipment).

Gary et al., 2008 indicates that while reference intervals based on the meta-analysis may be used, it still needs to be validated prior to usage by academic, research, pharmaceutical laboratories, and regulatory bodies. This involves transference and the usage of 20 collected samples for supposed healthy animals. A reference interval is thus recommended for adoption only when 2 of the 20 samples fall within the newly created reference interval, while when more than 3 of 20 fall outside the limit, the intervals are then considered unacceptable. Alternatively, it may be prudent to determine South African specific reference interval for locally bred animals, with the requisite number of breeding animals needed to meet statistical significance. Of the two methods, we would recommend the latter.

4.4 Conclusion

To understand possible toxic effects of tested drugs, among inbred Sprague Dawley (SD) rats, it is essential to establish reference intervals for an effective comparison. At present the available reference intervals have been published by international laboratories and likely not relevant for South African studies due to the influence of genetic drift. To accommodate for this, reference intervals can be established using large datasets from healthy control animals. However due to the expense of such studies, it may be possible to validate the international results by combining the results from smaller local studies using the biomedical statistical methodology known as meta-analysis. For this study we combined data from multiple studies, possibly from multi-site, to establish. The results from the said analysis was unable to show similarity of calculated reference intervals to the international reference intervals indicating that genetic drift has potentially occurred. As a result further studies are needed for the development of new reference intervals. Also the degree of genetic drift needs to be clarified through genetic analysis.

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