CHAPTER-3

HYPOTHESIS AND METHODOLOGY
3.1. Hypothesis:

The hypothesis of my study is that there exists relationship between NT pro BNP, TSH and obesity in hypothyroidism i.e. in obese individuals with recently diagnosed hypothyroidism without any history of thyroid and cardiac problems will have more N terminal Brain Natriuretic Peptide. These people will have risk for congestive heart failure.

The above main hypothesis is achieved through the following sub hypothesis

1. H₀ – In comparison with control group N-Terminal pro BNP will not be increased in case of hypothyroidism.

   Hₐ. Increased levels are seen in hypothyroid cases

2. H₀. The incidence of metabolic syndrome is not more in hypothyroid cases compared to euthyroids

   Hₐ - is that incidence of metabolic syndrome is more in subjects with hypothyroidism

3. H₀. There is no difference in levels of lipids in hypothyroid and Euthyroid cases

   Hₐ. is that the lipid levels are different in hypo and Euthyroid subjects

4. H₀. There is no correlation between the levels of lipids in case of obese and non obese people

   Hₐ. The levels of lipids in obese and non obese individuals are related

5. H₀ – N-terminal pro BNP concentration is not related in people with obesity and without obesity.

   Hₐ - NT pro BNP levels in obese and non obese subjects are related

6. H₀. Obesity and Incidence of Met S in related

   Hₐ is that Incidence of Met S is more in case of obese individuals.
3.2. Objectives:

- To screen for TSH levels in general public who visit tertiary care hospital as outpatients and to categorize the subjects into 3 categories; hypo, hyper and euthyroid subjects. Euthyroid subjects are taken as control group and hypothyroid subjects are taken as study group.

- To calculate the BMI in cases i.e hypothyroid patients to categorize obese and non-obese.

- To assess the risk associated with obesity and hypothyroidism i.e to estimate cardiac risk marker, N-Terminal Pro BNP in subjects who are obese and overweight with hypothyroidism.

- Both control and study group are assessed for metabolic syndrome (WHO guidelines)

Main objectives are further detailed as below-

- To estimate and study the N-Terminal pro BNP concentration in case of hypothyroidism.

- To study metabolic syndrome incidences in case of control group and cases.

- To study the levels of lipid profile parameters in cases and controls.

- To study the levels of obesity (BMI) - in cases and controls.

- To study and correlate the levels of N-Terminal pro BNP in subjects with obesity and without obesity.

- To study how N-Terminal pro BNP is associated with lipids in obese and nonobese subjects.

- To study the Incidence of metabolic syndrome in obese cases in comparison to nonobese subjects.
3.3. Method

- Selection of a General population of 2000 (n=2000), approximately 17% (n=517) of the general populations are known to present with hypothyroidism.

- Include hypothyroid subjects (n= 88). About 50% of subjects with hypothyroidism present with obesity (others we were not able to collect BMI and NT-pro BNP is not estimated).

- Estimate cardiac risk by means of the parameter NT-pro BNP in Obese & Non-Obese subjects.

3.4. Work Plan

- Adult individuals visiting Master Health Check (MHC) department of Vikram hospital, a tertiary care hospital Bangalore were included in the study as per the objective.

- The staff in the MHC department explain them about the MHC packages which the people like to take and whoever is taking the MHC package with NT-pro BNP as additional test were included in the study.

- The thyroid and cardiac history are collected for those individuals during phlebotomy.

- From the list of tests in the health check package the few of the following test parameters I have taken for my study they are blood sugar, Lipid profile, BMI, serum creatinine, TSH, metabolic syndrome, age, sex and NT-pro BNP.

- On the basis of levels of Thyroid Stimulating Hormone (TSH) the population is been categorized as hypo and Euthyroid subjects.
• BMI is calculated using height and weight taken from calibrated scales. This is done for both hypothyroid (cases) and Euthyroid subjects (control) so as to categorize them into obese and non-obese group.

• Lipid profile, Blood sugar and creatinine is measured as a part of organ function screening.

• As per WHO guidelines, presence of metabolic syndrome in those individuals was calculated.

• NT pro BNP is estimated for control group and hypothyroid study group

• The data of the individuals who are newly diagnosed for hypothyroidism with TSH >4.2micIU/l without any cardiac or thyroid history were collected and N-Terminal pro BNP is performed for only those individuals and were included in the study.

Inclusion criteria-

∧ Subjects between age 20 to 80 years both male and female.

∧ No Thyroid and cardiac history

∧ All recently diagnosed hypothyroid subjects (TSH >4.2micIU/l)

Exclusion criteria-

∧ All subjects with age <20 >80 years .

∧ With history of Thyroid and cardiac problems

∧ Subjects whose TSH levels are <4.2mic I U/l

Consent was taken in the form of permission letter to collect laboratory data from the vikram hospital management .
Estimation of N-Terminal pro BNP: This test has been done using immunoassay method on AQT 90FLEX analyzer. There is a formation of sandwich complexes between tracer antibodies and capture antibodies and measurement is done by the method of fluorescence. The concentration of antigen present in the blood to be measured is proportional to the fluorescence and the photomultiplier tube calculates the amount of photons. AQT 90FLEX has got its limit of detection 12 ng/L. The limit of detection has been determined to be 12 ng/l. Concentration up to 35000 ng/L can be reported using this analyser. Heparin blood is used for the test. Cut off value for the detection of the disease is 133 ng/l.


Estimation of TSH and other metabolites:

Fasting venous sample (3 ml) is collected in a gel vacutainer and the serum is been used for TSH and other metabolic parameters like serum glucose (GOD/POD), Total cholesterol (CHOD/POD), Triglycerides (GPO/POD), HDL cholesterol (enzymatic method), Serum creatinine (enzymatic method).

Estimation of Thyroid Stimulating Hormone (TSH)- TSH is estimated on a fully automated platform Minividas from Biomerieux using ELFA method.

When new lot of reagents is used the specifications must be entered into the instrument using master lot entry (MLE) data. Calibration has to be done after the MLE data entry using the calibrator provided in the kit and should be performed every 14 days. Calibration has to be checked using control provided in the kit. The results of TSH must be interpreted as part of a complete clinical profile. Measurement range of VIDAS TSH kit is up to 60 mic IU/ml and detection limit is 0.05 mic IU/ml.

All the other test parameters like glucose, cholesterol, Creatinine, triglycerides, HDL cholesterol are quantitatively measured using Vitros 250 micro slide dry chemistry analyzer from Ortho Clinical Diagnostics. The analyzer is been calibrated as per the
standard requirement given in the manual. All the tests are individually calibrated using the Vitros chemistry products calibrator kit and performance has been validated using performance verifier(PV1 and PV2).

Calibrator kit 1 is used for calibrating Glucose which is traceable to NIST SRM 917 and comparative method is AACC/CDC (Hexokinase). and Creatinine, which is traceable to NIST SRM 914 and comparative method is IDMS.

Calibrator kit 2 is used for calibrating Cholesterol which is traceable to NIST SRM 911 and comparative method is CDC Modified Abell-Endall. Triglycerides which is traceable to NIST SRM 1951 and comparative method is GPO spectrophotometric method.

HDL cholesterol which is traceable to NIST SRM 911 and comparative method is CRMLN designated comparison method.

**Procedure for Serum Glucose estimation:**

Vitros 250 analyzer from OCD is used for glucose assay. Cal kit 1 is used for calibration of the test and Glucose slides are used. Drop of serum is added into the glucose slide. The spreading layer will take care of the interferences, reaction takes place in the reagent layer. The dye formed is measured by the reflectance spectrophotometry method and it is proportional to the concentration of glucose in blood.

\[
\text{Beta D glucose + H}_2\text{O + O}_2 \rightarrow \text{GOD} \rightarrow \text{D-gluconic acid + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{4-aminoantipyrine + 1,7-dihydroxynaphthalene peroxidase red dye}
\]

<table>
<thead>
<tr>
<th>Method</th>
<th>Colorimetric</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitros system</td>
<td>250</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5 mins</td>
</tr>
</tbody>
</table>
At temperature | 37 deg (98.6 °F)
wavelength | 540 nm
specimen | 10 μL

Specimens Recommended: Serum, Heparin plasma

Specimens not Recommended: Plasma (EDTA, Sodium fluoride/potassium oxalate)

Special Precautions

• Highly lipemic specimens must be diluted before processing

• Centrifuge blood after clotting to avoid interfering substances like fibrin.

• Blood is centrifuged at 3000 RPM to get serum, centrifugation is done for ten minutes to have proper separation and clear serum done

• To get from plasma from heparin, mix the specimen then centrifuge at 3000 rpm for 10 minutes and remove plasma for the test

• Sodium fluoride/potassium oxalate plasma: To get plasma from this, centrifuge the specimen at 3000 rpm for 10 minutes

• Specimens are stored in air tight containers in the freezer after analysis and brought to R T and mix well before processing.

Measuring (Reportable or Dynamic) Range 20.0–625.0 mg/dl

Quality Control Procedure Recommendations:

• To check the calibration
• Process quality control material before or during patient sample analysis.
• To verify system performance, analyze control materials.
• As per the criteria given in NABL112.
• When the maintenance of equipment takes place.
• When QC results goes out of range range, identify the root cause, correct it and prevent it. Do not release patient results till the QC value is acceptable.

Expected levels for Glucose in fasting sample-74-106mg/dl

**Procedure for estimating Total cholesterol:**

The Total Cholesterol test is done on VITROS 250 dry chemistry system. Here the Cholesterol Slides and the Calibrator Kit 2 are used. Serum is dropped on the test, reaction occurs to produce a dye which is colored, the density of the colored dye is proportional to the concentration of cholesterol. The principle of measurement is reflectance spectrophotometry. The researcher Allain et al suggested this enzymatic method of cholesterol estimation.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>Colorimetric</th>
</tr>
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<tbody>
<tr>
<td>ANALYSER</td>
<td>VITROS250</td>
</tr>
<tr>
<td>INCUBATION</td>
<td>5MINS</td>
</tr>
<tr>
<td>TEMPERATURE</td>
<td>37 DEGREES</td>
</tr>
<tr>
<td>WAVELENGTH</td>
<td>540NM</td>
</tr>
<tr>
<td>SAMPLE</td>
<td>5.5MICRO LITRE</td>
</tr>
</tbody>
</table>

lipoprotein   **TX100**   cholesterol + cholesterol esters + proteins

cholesterol esters + H2O cholesterol ester **hydrolase** cholesterol + fatty acids

cholesterol + O2  **cholesterol oxidase** cholest-4-en-3-one + H2O2
H$_2$O$_2$ + leuco dye \textit{peroxidase} dye + 2H$_2$O

\textbf{Specimens Recommended}: Serum, Heparin plasma but EDTA plasma and fluoride plasma cannot be used

\textbf{Special Precautions}

- Highly lipemic samples have to be diluted
- Centrifuge the samples only after clotting to avoid interference from fibrin
- To get serum or plasma centrifuge the specimen at 3000rpm for 10 minutes

Specimens are stored in air tight containers and stored in freezers then brought to room temperature and mix well before analysis.

- Check reagent quantities whether it is sufficient for the planned workload.

\textbf{Measuring (Reportable or Dynamic) Range} 50.0–325.0 mg/dl

(Ref: NCEP guidelines)

Less than 200 mg\% is desirable

200 mg\% to 239 mg\% is termed as borderline high

More than 240 mg\% is the value for high risk

\textbf{Procedure for estimation of HDL Cholesterol}

Direct HDL is performed on Vitros 250 analyser using Vitros dHDL slides and calibrator kit 25 using micro slide technology. The measurement principle is reflectance
spectrophotometry. As discussed earlier in case of total cholesterol and HDL slide also has multilayers with the polyester support.

Blood sample is placed on the microslide. HDL is separated from other lipoproteins and the surfactant Emulgen B-66 responsible for breaking cholesterol and esters from the HDL-lipoprotein complex. Cholesterol ester hydrolase hydrolyses HDL cholesterol ester to cholesterol. This cholesterol oxidized to cholestenone and hydrogen peroxide. \( \text{H}_2\text{O}_2 \) oxidises leuco dye to form a colored dye by peroxidase. HDL concentration in the sample is directly proportional to the color of the dye formed.

<table>
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<td>Vitros system</td>
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</tr>
<tr>
<td>Time of incubation</td>
<td>5mins</td>
</tr>
<tr>
<td>TEMPERATURE</td>
<td>37 degrees</td>
</tr>
<tr>
<td>WAVELENGTH</td>
<td>670nm</td>
</tr>
<tr>
<td>SAMPLE</td>
<td>10( \mu \text{L} )</td>
</tr>
</tbody>
</table>
• Specimen has to be centrifuged at 3000 rpm for about 10 mins to get serum and plasma.

**Measuring (Reportable or Dynamic) Range** 5.0–110.0 mg/dl

**Reference Interval** <40>60 mg/dl (NIH guidelines)

**Estimation of Triglycerides:**

The estimation of triglycerides are performed on Vitros 250 dry chemistry system using triglyceride slides and calibrator kit 2. This Vitros 250 analyzer works on the principle of microslide technology by enzymatic method explained by Spayd et al.

Serum sample is dropped on the microslide and dispersed by the layers of the slide. The Triton X100 is used to splits triglyceride molecules from lipoprotein complexes in the serum. The lipase hydrolyses triglycerides to form glycerol and fatty acids. Glycerol diffuses to the reagent layer, where it is phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of L-α-glycerol-phosphate oxidase, L-α-glycerophosphate is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involves the oxidation of a leuco dye by hydrogen peroxide, catalyzed by peroxidase, to produce a dye. The concentration of triglycerides is proportional to the density of the dye. This is measured by the principle of reflectance spectrophotometry.

**Specimens recommended:** Plasma collected from heparin and serum

**Specimens not recommended:** Plasma from EDTA blood

The person who is for triglyceride testing has to fast overnight. Once the blood is collected then that has to be centrifuged at 3000 rpm to get serum or plasma

Special Precautions: All the equipment’s must be kept soap and glycerol free.

**Required calibrators:** Calibrator kit 2 for Vitros.

**Calibrate:**
• If there is change in the reagent lot.
• After service or preventive maintenance
• As per the company recommendations
• When the internal QC is out of range

**LDL cholesterol and VLDL cholesterol**

LDL and VLDL are calculated by the following formula.

\[
LDL = \text{Cholesterol} - \text{HDL cholesterol} - \text{VLDL}
\]

\[
VLDL = \frac{\text{TRIG}}{5} \text{ in mg/dL}
\]

The above calculation for LDL is not valid if TGL levels are >400mg/dl or for individuals who has got type III hyperlipoproteinemia.

**Normal range –LDL cholesterol**

<table>
<thead>
<tr>
<th>LDL Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100</td>
<td>Desirable, indicates less chance of cardiovascular events</td>
</tr>
<tr>
<td>100 to 129</td>
<td>Borderline LDL level, risk of getting cardiovascular diseases with symptoms</td>
</tr>
<tr>
<td>130 to 159</td>
<td>Borderline high LDL level, suggestive of increased CVD risk</td>
</tr>
<tr>
<td>160 to 199</td>
<td>High LDL level, suggestive of very high chance of getting cardiovascular events</td>
</tr>
<tr>
<td>&gt;200</td>
<td>Extremely high levels of LDL with very high chance of developing cardiovascular problems.</td>
</tr>
</tbody>
</table>

Ref: NIH and NCEP guidelines
**Estimation of serum creatinine levels:**

The creatinine test is performed on Vitros 250 chemistry system. Creatinine dry chemistry slides and calibrator kit 1 is used here.

Serum sample is added on to the Vitros creatinine slide which has got reagent .The leuco dye formed at the end of the reaction is measured by the reflectance photometric principle. After adding specimen incubation of the will take place for the reaction to take place. After the oxidation of the endogenous creatine the change in the reflection density is estimated in two time points .The concentration of creatinine is proportional to the difference between the two densities .

<table>
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<td>670nm</td>
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<tr>
<td>SAMPLE</td>
<td>6microlitre</td>
</tr>
</tbody>
</table>

Specimen type: Serum, Heparin plasma. After collection, centrifuge the sample at 3000rpm and separate the serum within 4 hours.

Patient Preparation: Randomly we can collect the sample for this test.

Calibration is done using Vitros Chemistry Calibrator Kit 1

**Calibrate:**

- If there is change in the lot
- After service or preventive maintenance.
• As per the requirement given in the manual

• Whenever internal QC is out of range.

Validation of calibration has to be done using performance verifiers

**Procedure for estimation of TSH:**

Thyrotropin or Thyroid stimulating hormone (TSH) is measured using autoanalyser called Minividas which measures quantitatively by using Enzyme Linked Immuno Fluorescent Assay (ELFA) technology. Analyzer calculates and gives the results by making use of calibration graph stored in it and the same can be printed

Specimen: Serum or heparin plasma is used, EDTA plasma cannot be used.

Specimen stability is 48hrs at 2-8 degrees. Before each new lot of reagents Master lot Entry has to be done to feed in the data.

Calibration is done using calibrator provided in the kit and checked by the quality control. The measurement range is upto 60 micIU and detection limit 0.05micIU.

**Definition obesity in terms of BMI:**

As per WHO information the excess or abnormal accumulation of fat which lead to health risk is called overweight or obesity and can be measured in terms of body mass index. This is the calculation here the height in meter square is divided by the body weight in kilograms. The body mass index 30 or more than that is termed as obese and >25. A person with a BMI equal to or more than 25 is categorized as overweight.

Inclusion criteria- All subjects aged between 20 to 80 years without history of renal, cardiac and thyroid problems and with recently diagnosed sub clinical hypothyroidism (TSH >4.2mic IU/ml) are included for the study. Renal function is evaluated by serum creatinine estimation.

Categorization into Hypo, Hyper and Eu thyroid is been done based on the normal reference range given for TSH The value below the range is Hyperthyroid and above the range is Hypothyroid( Scanlon M F,TOFT A.D 1996).
Normal range for Thyroid stimulating hormone is 0.4 to 4.0 µIU/l. TSH concentrations rose with advancing age as studied by Hollowell J G et al 2002. But as per the third National Health and Nutrition Examination survey data about 80 percent of the individuals showed thyroid stimulating hormone levels less than 2.5 µIU/l.

Method used here was Enzyme Linked Fluorescent Assay and the analyzer used was Minividas immuno assay analyzer from Biomeriux.

BMI and NT pro BNP test has been conducted for all the subjects who passed the eligibility criteria. Control group for hypothyroidism is those with normal TSH and for obese group it is non-obese subjects. Hypothyroid patients (n=88) will be assessed for BMI according to the objective. Height and weight of the subjects are collected and Standard methods for measuring BMI will be used. BMI is measured in terms of kg/m².

As per the classification based on the global database adapted from WHO guidelines 1995, 2000 and 2004, the population of study has been categorized as below.

Group A are underweight subjects with a BMI less than 18.

Group B are healthy subjects with a BMI 18.5 - 25

Group C are overweight subjects who have BMI between 25 - 30

Group D with BMI more than 35.

Subjects of category of group C or D i e (>25 kg/m²) will be included in the study for assessment of cardiac marker NT-pro BNP to fulfill the objective. The subjects included will be tested for NT-pro BNP with informed consent. NT-pro BNP will be measured on AQT 90 FLEX, a fully automated immunoassay analyzer. The quantitative result for NT pro BNP will be given by the analyzer in 10 mins. AQT90 FLEX works with the principle of dry chemistry technology from the company Radiometer. There is no need for sample preparation prior to the analysis. This instrument provides précised results with unmatched operator safety and hence this is an ideal instrument which can be kept in both laboratory and point of care environment. The value less than 133 ng/l is considered as negative for N-Terminal pro BNP. Using statistical analysis of the data
collected the association between N-Terminal pro BNP with thyroid stimulating hormone with respect to body mass index has been explained.

WHO guidelines was used for deciding whether the subject included for the study has got metabolic syndrome or not. The following parameters were taken in the present study such as Blood glucose >100mg/dl ,Triglycerides (TG)more than150mg/dl and HDL cholesterol <34.7mg% for males and <39% for females and BMI>30kg/m2. These data are also analyzed using statistical tools along with other parameters to arrive at a significant outcome.

**Statistical software:** The name of the software used for statistical analysis of the collected data is statistical package for social sciences (15). Data are presented in the form of text, graph, diagrams etc[ Bernard Rosner (2000), Robert H Riffenburg (2005), Sunder Rao P S S, Richard J(2006), Suresh K.P. and Chandrasekhar S (2012)].

**Statistical methods:**

Statistical methods used for explaining, describing and organising the collected data. The present study used some of the descriptive statistics and inference is made. Numerical and graphical calculations are used for summarising the collected data from that population. These measurements are described in terms of Mean and Standard Deviation. To attain the inference of the study or to test the hypothesis of the study, inferential statistics is used. Significance of the research work is measured in terms of levels of significance.

The following are the assumptions on data

1. Normal distribution of dependent variables,

2. Random sampling with samples from independent cases.

When there is normal distribution of data Student t test is used to test the hypothesis. Chi squared test is always used to test the null hypothesis. This is used to compare the observed data and obtained data. Here null hypothesis says there is no difference between observed and obtained data.
1. Sample Size estimation

Sample size for the study has to be calculated statistically for the population of study.

In that ME is the margin of error, measure of precision N is population size

n is Sample size, $\sigma$: Standard deviation, $z$: Critical value based on Normal distribution at 95% Confidence Interval and

Standard deviation: $SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$

2 Chi square tests: To find out the association between the 2 different variables in a population the statistical tool Chi square test of independence is used. Here independence means that there is no relationship between the two variables.

$\chi^2 = \frac{\sum (O_i - E_i)^2}{E_i}$, Where Oi is Observed frequency and Ei is Expected frequency

With (n-1) df

The Assumptions of Chi-square test

Chi squared test can be used when the below mentioned conditions are met.

- The samples selected should be simple random in a given population of study

- Sample size should be large otherwise if the small size samples may give type II error in inferring results. Population should be 10 times bigger than the sample. When this condition is not passed, Fisher Exact test or Yates correction is used.

3. FISHER EXACT TEST: This is a more accurate statistical method than Chi squared test. This is used to find out whether different treatment yield different results or yield in cross tabulation. Null hypothesis is result does not affect result or the yield. Both are independent and then null hypothesis has to be rejected when p value found to be less.

4 STUDENT TEST (Two tailed, independent)
Assumptions:

Random allotment of subjects to one of the 2 groups

Test statistic should follow normal distribution having equal variances

Hypothesis testing for comparing two independent groups are as follows

When two groups are equal then Ho: $u_1 = u_2$, if 2 groups are unequal then Ha: $u_1 \neq u_2$

Null hypothesis has to be rejected and alternate hypothesis has to accepted when the p value less than 0.05 Or this confirms statistically the differences in the means are significant. The test statistic is given here

t-test: Two sample assuming equal variances

\[ S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}} \]

In all work with two-sample t-test the degrees of freedom or df is:

\[ df = n_1 + n_2 - 2 \]

The formula for the two sample t-test is:

\[ T = \frac{\overline{X} - \overline{Y}}{S_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \]

t-test: This test is used for two sample assuming unequal variances

\[ T = \frac{\overline{X} - \overline{Y}}{\sqrt{\frac{S_X^2}{n_1} + \frac{S_Y^2}{n_2}}} \]
Note in this case the Degree of Freedom is measured by

\[ df' = \frac{\left( \frac{s_1^2}{n_1} + \frac{s_2^2}{n_2} \right)^2}{\left( \frac{s_1^2}{n_1} \right)^2 + \left( \frac{s_2^2}{n_2} \right)^2} \frac{n_1 - 1}{n_2 - 1} \]

and round up to integer.

Method of interpretation: While hypothesis testing reject null hypothesis and accept alternate hypothesis when the p value is less than 0.05 and p value is more than 0.05, no need to reject null hypothesis. The level of significance was reported in terms of p value. During the hypothesis test the p value found to be >0.05 then there is no evidence to reject null hypothesis.

5. Significant figures

P value: 0.05 < P < 0.10 suggests significant

P value: 0.01 < P < 0.05 suggests moderate significance

P value: P > 0.01 suggestive of high significance