Original Article

Neutrophil Lymphocyte Ratio and Mean Platelet Volume As Predictors for Coronary Artery Disease

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Abstract

Coronary artery disease is a progressive inflammatory disease with atherosclerosis playing pivotal role in its etiology. Neutrophil lymphocyte ratio(NLR) is increased in inflammatory disease and an independant predictor of adverse cardiac events. Large platelets are metabolically and enzymatically more active with great prothrombotic potential and thus resulting in adverse cardiovascular events.

The purpose of this study was to find whether NLR and MPV can be used as biomarkers for predicting CAD in normal individuals without any known comorbidities.

Hence a cross-sectional study which includes 80 normal healthy volunteers was conducted in the Department of Pathology and Department of Biochemistry in our institution.

Keywords: NLR; MPV; CAD.

Introduction

According to the World Health Organization (WHO) Coronary Artery Disease caused 17.5 million deaths worldwide.[1] Current estimates from various parts of India indicate a prevalence of Coronary Artery Disease (CAD) in human beings to be between 7-13% in urban and 2-7 % in rural areas.[2]

CAD is a progressive inflammatory disease with atherosclerosis playing a pivotal role in its etiology.[3] Gillum at al in his study showed that platelets and white blood cells express and secrete inflammatory mediators. Because of the inflammatory nature of CAD, both platelets and subtypes of WBC have been investigated by many studies for years.[4]

Previous study done Duffy et al proved that the combination of neutrophil and lymphocyte parameters has a better prognostic value in inflammatory diseases like CAD than each parameter separately.[5]

Papa et al[6] demonstrated neutrophil lymphocyte ratio (NLR) , an independent predictor of adverse cardiac events in CAD patients. Similarly Kalay et al[7]found NLR to be a predictor of the progression of atherosclerosis in coronary arteries.

Increased inflammatory cytokines also influence

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megakaryocytopoiesis.[8] Larger platelets are metabolically and enzymatically active with greater prothrombotic potential and thus resulting in adverse cardiovascular events. Mean platelet volume (MPV) is most commonly used to measure platelet size, which is a potential marker of platelet reactivity and CVD.[9]

In recent years, a strong interest has been drawn to these indices given that NLR and MPV may provide an independent information on pathophysiology, risk stratification and optimal management.[10]

Earlier studies also shown that approximately 90% of individuals with CAD have at least one antecedent traditional risk factor like smoking, hypertension etc[11] The close association between the traditional risk factors, atherosclerotic burden and risk for clinical CAD among middle-aged adults, allows for a single strategy of absolute risk assessment using the Framingham Risk Score 1. To identify candidates for medical therapy and 2. To encourage therapeutic lifestyle changes.[12]

Framingham score being a proven score, this study aims to use NLR, MPV as supportive predictors in assessing the risk of developing CAD. Hence my study is to assess the risk of CAD in normal individuals using the Framingham score and to correlate it with NLR and MPV.

Review of Literature

Coronary Artery Disease is a chronic inflammatory disease, caused by the remodeling and narrowing of the coronary arteries. Globally, CVD led to 17.5 million deaths in 2012. More than 75% of these deaths occurred in the developing countries according to a WHO report on 2014.

Its etiopathogenesis is complex and affected by various risk factors environmental factors like physical activity, smoking, and diet; and genetic factors.[13,14]

Atherosclerosis is the silent, progressive chronic inflammatory process which is characterized by accumulation of lipids, fibrous materials, and an inflammatory molecules in the walls of the large arteries.[15-18] Previous studies show the early involvement of the monocytes or macrophages, the most prominent cellular component of the innate immunity during atherogenesis.[19] Efflux of LDL molecules into the sub endothelial spaces of coronary arteries take place which are later oxidized by certain agents.

These modified lipoproteins interact with scavenger receptors of macrophages and send proinflammatory signals and thus these oxidized LDL particles are potent chemotactic molecules. Oxidized phospholipids derived from modified lipoproteins may also drive inflammation which induce vascular and intercellular adhesion molecules.[20] The monoctyes get attached to the activated endothelial cells by leukocyte adhesion molecules and differentiate into macrophages in
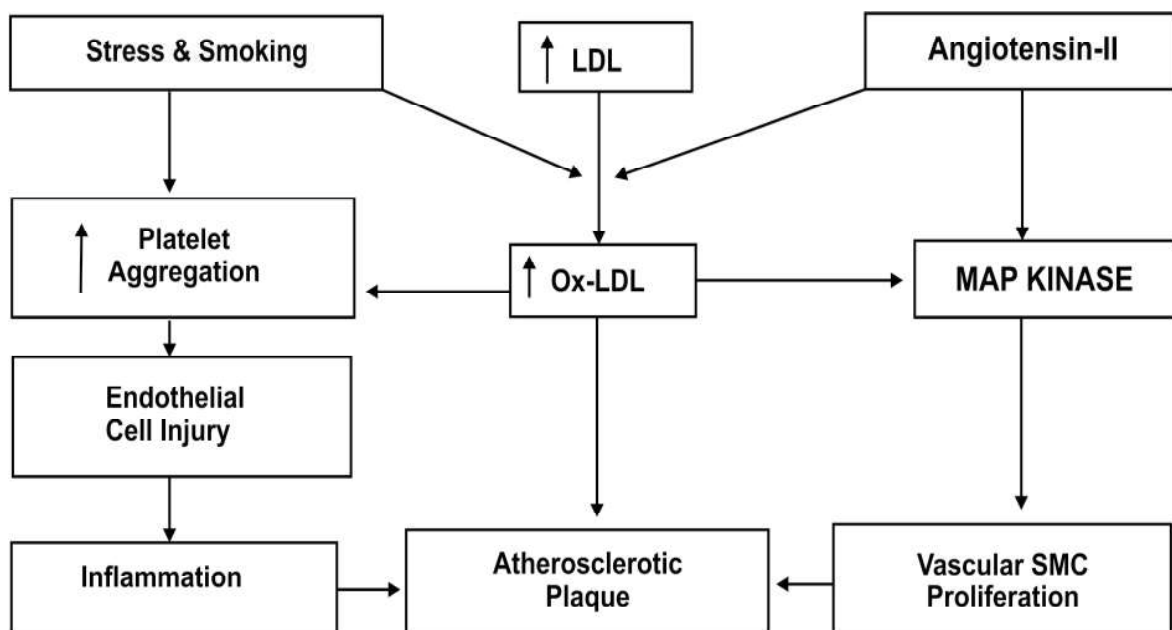

Fig. 1: Pathogenesis of atherosclerosis.

the intima media.[21]

These macrophages bind to oxidized LDL to become foam cells which release cytokines such as interleukins and tumor necrosis factor. The final result of this process is the formation of first typical atherosclerotic lesion, ie, the fatty streak.[15]

This process continues with the migration of smooth muscle cells from the medial layer into the intimal layer of the artery, resulting in the conversion of fatty streak into a more complex lesion[15] by producing an extracellular matrix creating a fibrous cap around the original fatty streak. Foam cells present inside the fibrous cap die and release lipids that accumulate in the extracellular space and form a necrotic core[22] The result of this process is the formation of second atherosclerotic lesion, the fibrous plaque.
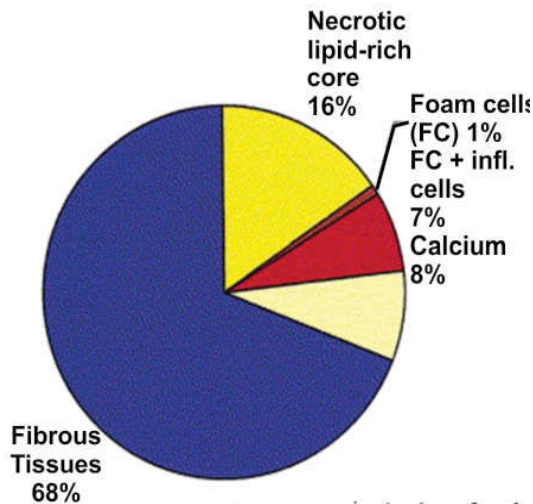

Fig. 2: Composition of atherosclerotic plaque.

The thickness of the fibrous cap is the key for determining the integrity of the atherosclerotic plaque. The two types of plaque can be defined depending on the balance between formation and degradation of this fibrous cap-stable and unstable. The stable plaques has intact, thick fibrous cap composed of smooth muscle cells and matrix, rich in type I and type III collagen fibres. where as vulnerable plaques have a thin fibrous cap made up of few or no smooth muscle cells and mostly of type I collagen but have abundant macrophages and prothrombotic molecules.[23]

These plaques are highly prone to erosion, exposing the core of the plaque to circulating coagulants causing thrombosis. Intraplaque hemorrhage which appears to be occur when vasa vasorum invades the intima from the adventitia,

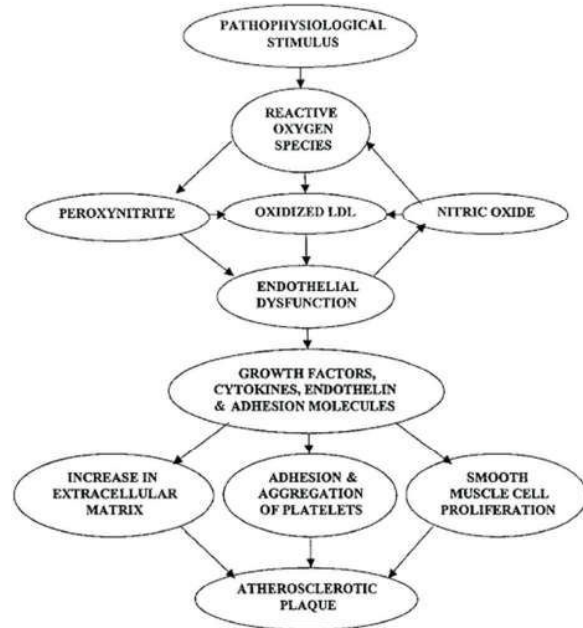which is also a potential contributor of progression of atherosclerosis.[24]

Fig. 3: Factors contributing to progression of atherosclerosis.

So atherosclerosis is basically an inflammatory disease and thus elevated levels of systemic inflammatory markers have been associated with the incidence of coronary artery disease.[25]

Pitsavos et al. study also shown that chronic, low grade inflammation is seen in diabetes mellitus, hypertension, metabolic syndrome, obesity, smoking, and other lifestyle habits, which are all the risk factors for development of CAD which is measured by the WBC count.[26-30] Studies in different populations have shown that increased level of WBC can be linked with incident CAD. This may be due to the cellular response of the blood components which might be mediated through endothelial dysfunction. Inflammation modifies the endothelial function and this causes an inability of the endothelium to produce nitric oxide and prostacyclin, which results in the depletion of vasodilator, antithrombotic and anti-atherogenic properties of the vascular endothelium. In addition, stimulated leukocytes have altered rheological properties and an increased capacity to adhere to vascular endothelium resulting in capillary leukocytosis followed by increased vascular resistance.[26]

Vasan R.S.et al. have reported the relationship between inflammatory biomarkers and the risk for development of coronary artery disease, in apparently healthy individuals as well as in patients

with coronary artery disease. But the clinical utility of the biomarker for risk prediction depends upon its ease, cost, practicability, reproducibility of measurement and its ability to add to the predictability of existing biomarkers such as those included in the Framingham algorithm.[31] Neutophil lymphocyte ratio and MPV are two of such biomarkers.

Neutrophil Lymphocyte Ratio is the ratio of absolute counts of neutrophils and lymphocytes, which is an effective biomarker in the stratification and prognosis of CAD.[32] The NLR is a derived marker, simple, inexpensive,easily available, and found itself to be a good predictor for other adverse cardiovascular outcomes as well.[33-35] It reflect an imbalance in the inflammatory cells and the role of activated neutrophils in atherogenesis.

C.D.Lee et al. have also evaluated the predictive ability of WBC counts. Increased WBC count, a marker for inflammatory state, was proven to be a predictor for long-term cardiovascular mortality. Recent studies suggest some specific leukocyte populations as predictors for cardiovascular risk and that lymphopenia seen in heart failure was associated with poor outcome.[36] Shah N et al. in a cohort study have shown that NLR can independently predict CHD mortality in an asymptomatic general population.[32]

The dominance of neutrophils over lymphocytes is indicative of a deeper imbalance in the immunologic response. Hence increased NLR will result in the activation of interleukin-17[37], which is a cytokine produced by the T-helper 17, a subset of T-helper cells. The naive T cells differentiate into Th17 by IL-6[38], produced by the macrophages. It also stimulates the production of more proinflammatory cytokines like tumor necrosis factor (TNF)-α and IL-6[39,40]and regulates the tissue infiltration by neutrophils and myocyte apoptosis, that initiate many other pathophysiological pathways like oxidative stress and hypercoagulability.

NLR is significantly increased in individuals with diabetes and smokers, which are two of the traditional risk factors for CAD as there is a significant relationship between systemic inflammation and diabetes mellitus. Many studies have reported that chronic, low grade subclinical inflammation leads to the development of insulin resistance, which later results in clinically overt diabetes mellitus.[41] Risk factors of diabetes mellitus includes obesity, smoking and physical inactivity are also associated with chronic inflammation. Similarly cigarette smoke constituents also induce immune response. Reactive Oxygen Species are produced

in the burning cigarette which are not removed by cigarette butt filters.[42] They damage the epithelial cells lining the airways, by peroxidation of lipids and other cell membrane constituents, induces DNA damage.[43] It activates intracellular signaling cascades that lead to inflammatory gene activation like interleukin-8 and tumor necrosis factor-alpha (TNFα).[44] The secretion of these inflammatory mediators promotes chronic inflammation.

Neutrophilia or lymphopenia results in high NLR while lymphocytosis or neutropenia results in low NLR. High NLR points to a predominance of inflammatory mediators in the body .The main role of neutrophilia in CAD is through the secretion of various inflammatory mediators such as elastase, myeloperoxidase, and oxygen free radicals which causes tissue damage. Lymphopenia may result from the increased steroid level due to CAD induced stress and increased apoptosis triggered by increased inflammation thus causing an increase in NLR in CAD patients.[45,46] Both increased number of neutrophils and decreased lymphocytes are the risk predictors for future cardiovascular adverse events. Therefore, NLR integrates the predictive risk of the two leukocyte subtypes into a single risk factor. The result of eight cohort studies involving patients undergoing myocardial revascularization or coronarography, showed that high NLR increased the risk of cardiovascular and mortality by 2 times.[47]

Patrice Forget et al. have identified that the normal NLR values in an adult, non-geriatric, population in good health are between 0.78 and 3.53[48]. In Chennai, Shiny et al, reported a NLR of 1.5 ± 0.41 among healthy non-glaycaemic individuals.[49] However the reference values for NLR vary with age and ethnicity. Studies by Uthamalingam et al,B Azab et al., have categorized their patients according to NLR intervals like tertiles, quartiles, and quintiles.[50,51] while others like Y.Ohno et al.,I Bhatti et al.[52,53] have used definite NLR cut-off points like NLR ≥ 2.5, NLR ≥ 2.7, NLR ≥ 3, NLR ≥ 4, NLR ≥ 5 Hence Neutrophil Lymphocyte Ratio can be used as a biomarker for CAD. George S.et al.have shown that Mean Platelet Volume, is another biomarker for prediction of risk in CAD and measures the average size of the platelets in peripheral blood.[54] An increased MPV indicates large platelets resulting from increase in young platelets in circulation.[55]

Platelets secrete and express a large number of substances that are crucial mediators of coagulation, inflammation, thrombosis, and atherosclerosis[56] and thus play a crucial role in the atherothrombosis process. Hence measurement of platelet activity and

aggregation can provide prognostic information for the risk of cardiovascular events.

Larger platelets are more active metabolically and enzymatically than smaller platelets, containing more prothrombotic material like thromboxane A2 and B2 per unit volume and glycoprotein IIb–IIIa receptor expression. They show greater aggregability in response to ADP and decreased inhibition of aggregation by prostacyclin. Larger platelets are denser containing more of α-granules, which release prothrombotic substances, like platelet factor 4, P-selectin, and platelet-derived growth factor, a chemotactic and mitogenic factor contributing to vascular neointimal proliferation.[57-64]

MPV that is an indicator of platelet sizes, it is routinely used as a cheap assessment tool that implemented both hospitalized patients and outpatients. The normal range of MPV is 8.9 ± 1.4 in a study by Demirin et al.[65]

CAD risk is analysed using Framingham Risk Score (FRS) which has evolved into a validated means of predicting cardiovascular disease (CVD) risk in asymptomatic patients all over the world. Input variables are easily obtained from office history, physical examination, and simple laboratory evaluations.[66] FRS is the most applicable method for predicting the person's chance of developing cardiovascular disease (CVD) in long term.

The FRS considers six coronary risk factors, including age, gender, total cholesterol (TC), high density lipoprotein cholesterol (HDL), smoking habits, and systolic blood pressure.[67]

The 10-year risk percentage derived can then be used to initiate lipid-lowering therapy for primary prevention. Risk is considered low if the FRS is less than 10%, moderate if it is 10% to 19%, and high if it is 20% or higher.

Because this risk score gives an indication of the likely benefits of prevention, it can be useful for both the patients and clinicians deciding whether lifestyle modification and preventive medical treatment and for patients education by identifying men and women at increased risk for future cardiovascular events.[68] Mahmood S.S. et al. have shown that the major risk factors identified in this score apply universally to all racial and ethnic groups.[69]

Earlier studies have shown that approximately 90% of individuals with CAD have at least one antecedent traditional risk factor like smoking, hypertension etc.[11] The close association between traditional risk factors, atherosclerotic burden and risk for clinical CAD in middle-aged adults allows for a single strategy of absolute risk assessment using the Framingham Risk Score[1] to identify candidates for medical therapy and[2] to encourage therapeutic lifestyle changes.[12]

All the major risk factors of CAD included in Framingham Score has an inflammatory pathology and thus the severity of CAD could be predicted using inflammatory markers like NLR and MPV .

Aim

- To assess the risk for CAD using Neutrophil Lymphocyte Ratio and Mean Platelet Volume.

Objective

- To assess the NLR and MPV in the study population.
- To assess Framingham score for risk of development of CAD in the study population.
- To correlate the NLR and MPV with the risk of development of CAD assessed by the Framingham score.

Materials and Methods

Study Design: Cross-Sectional Study

Study Centre: Department of Pathology and Department of Biochemistry In Our Institution.

Study Period: June – Oct 2018

Study Population: 80 normal individuals aged between 35 to 70, without any known co morbidities

Sample Size Calculation: For an expected incidence (p) of 13% of CAD cases with Z value of 1.96 at 95% confidence interval, and with limit of accuracy(L) at 10% of p (relative precision)the sample size required was 83.

q=1-p=0.7

The sample size required for the study was calculated as follows.

$$n = \frac{1.96 \times 1.96 \times 0.31 \times 0.7}{0.1 \times 0.1}$$

With an expected non response rate of 10%the required sample size was estimated at 90.

But only 80 samples could be taken.

Study Group

Inclusion Criteria: Normal individuals aged between 35 to 70 years.

Exclusion Criteria: Individuals with any known co morbid illness.

Investigations

Biochemical Investigations

Sample Collection: 3ml of venous blood will be collected and serum is separated by centrifugation for 10 mins. The serum will be aliquoted into 2 eppendorfs and stored at the temperature of -20 degrees for further investigations.

Fasting Plasma Glucose

Method: GOD/POD method (enzymatic, end point analysis).

Principle: Glucose present in the plasma is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid with the liberation of hydrogen peroxide, which is converted to water and oxygen by the enzyme peroxidase (POD). 4-aminophenazone, an oxygen acceptor, takes up the oxygen and together with phenol forms a pink coloured chromogen which can be measured at 515 nm.

Serum Creatinine

Method: Modified Jaffe's method.

Principle: Creatinine reacts with sodium picrate in the presence of an alkali to produce yellow orange creatinine picrate complex that is measured colorimetrically at 520 nm.

Urea

Method: UV-GLDH method

Principle: Urea is hydrolysed in the presence of urease to produce ammonia and CO2. The product ammonia combines with 2-oxoglutarate and NADH in presence of GLDH to yield glutamate and NAD. The decrease in absorbance is due to decrease of NADH concentration in unit time is proportional to the urea concentration (340nm).

Chief Investigations

Lipid Profile

Triglyceride (TGL):

Method: GPO-PAP (End point)

Principle: The triglycerides present in the serum are catabolized into glycerol and fatty acids by Lipoprotein Lipase, which is converted to glycerol-3-phosphate in the presence of glycerol kinase. It is acted upon by glycerol-3-phosphate oxidase to form hydrogen peroxide. This together with a phenolic compound, TBHBA and 4-aminoantipyrine in the presence of peroxidase gives a blue purple colour complex. The intensity of the blue purple colour is measured at 505nm (490-550 nm).

HDL:

Method: Direct. Enzymatic colorimetric

Principle: This method depends on the properties of a detergent which is solubilizes only the HDL, so that the HDL-c is released and react with the cholesterol esterase, cholesterol oxidase and chromogens to give the colour. The non HDL lipoproteins, LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to the absorption of the detergents on their surfaces, which is measured at 578nm.

Mean Platelet Volume

Method: Impedance Automated Pulse Analyzer

Principle: Laser Light Is Used. The Diluted Blood Specimen passes through a steady stream ,through which a beam of laser light is focused, as each cell passes through the sensing zone of the flow cell which scatters the focused light. The scattered light is then detected by a photo detector and converted to an electric impulse. The number of impulses generated is directly proportional to the number of cells passing through the sensing zone in a specific period of time.

Neutrophil Lymphocyte Ratio

Method: Autoanalyzer

Neutrophil lymphocyte counts were measured using an autoanalyser. NLR was calculated by dividing the neutrophil count with the lymphocyte count.

Framingham risk score

1. Age (in years)
2. Gender (male/female)
3. Total cholesterol (mmol/L)
4. HDL cholesterol (mmol/L)
5. Smoker (yes/no)
6. Diabetes (yes/no)
7. Systolic blood pressure (mm Hg)
8. Is the patient treated for high blood pressure? (yes/no)

Others

Measurement of BMI:

BMI = Weight in kgs/height in m2

Blood Pressure: Measured by a standard protocol:

Blood pressure is measured in sitting posture with feet rest, arm rest and back rest on the left arm using sphygmomanometer.

- The blood pressure is measured twice at an interval of 1 minute, mean of two readings considered
- The subject is diagnosed as hypertensive if systolic blood pressure > or = 140mmHg, Diastolic blood pressure > or =90mmHg and if he is on anti-hypertensive medication.

Data Collection Procedure and Instruments Used: Data collection has been done using standard proforma and done by the principal investigator. All the biochemical analyses will be performed using automated (alpha - IMMUCHEM) and semi-automated (MERCK) clinical chemistry analyzer. All anthropometric measurements are done using a plastic measuring tape.

Quality Control: All biochemical analysis will be done with adequate internal quality checks and within run and between run CV (Coefficient Variation) will be maintained.

Confidentiality: Informed consent will be obtained from all the patients. Confidentiality and safety of all the subjects has been maintained.

Statistical Analysis: Statistical analysis will be performed using spss 20.

- The groups were compared using unpaired t test.
- To assess the diagnostic value of the parameters. Receiver Operating Characteristic(ROC)the area under the curve (AUC) will be calculated.
- p value <0.05 will be considered statistically significant for two tailed test.

Observation and Results

These are the observations obtained from the cross-sectional study trying to correlate the inflammatory markers Neutrophil Lymphocyte Ratio and Mean Platelet Volume with the risk assessment algorithm of CAD-Framingham risk score.

Age Distribution

Mean age of the males is 48.37 (maximum age-58, minimum age-33).

Mean age of females is 43.731(maximum age-57, minimum age-32).

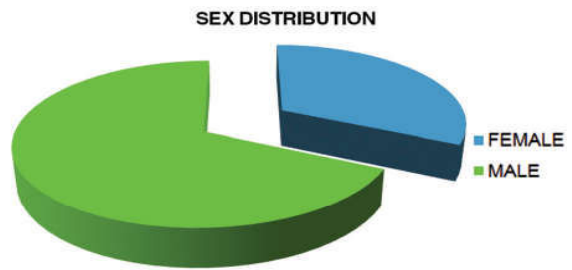| Sex Distribution		
Sex	Frequency	Percentage
Female	26	32
Male	54	68
Total	81	100

Fig. Neutrophil Lymphocyte Ratio in Males.

Mean NLR is 1.54
Range of NLR is 0.79 – 3.00
Correlation between NLR and the framingham risk score in males.

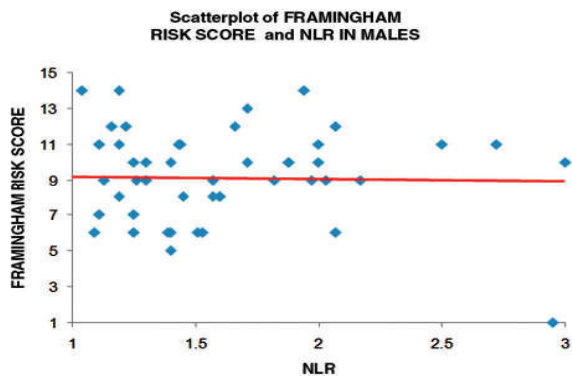| Pearson Correlation	-0.24
p Value	0.867

Fig. There is no significant correlation between NLR and Framingham risk score in the males.

Neutrophil lymphocyte ratio in females.
Mean NLR is 1.762
Range of NLR is 0.92 – 4.17
Correlation of neutrophil lymphocyte ratio and framingham score in females.

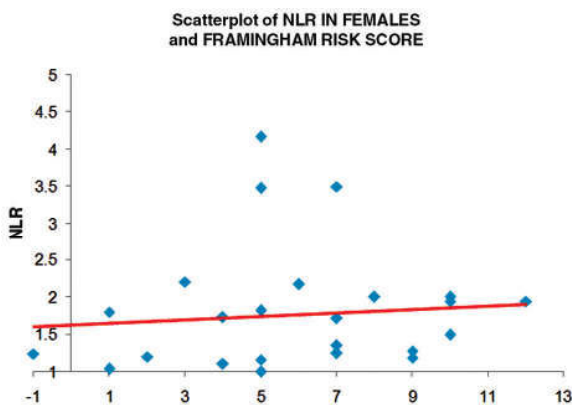| Pearson Correlation	0.90
p value	0.633

Fig. There is no significant correlation between NLR and Framingham risk score among females.

Mean platelet volume in males

Mean MPV in males is 9.763

Range of MPV in males is 7.2 – 12.2

Correlaton between mean platelet volume and framingham risk score in males.

Pearsons Correlation	0.267
p value	0.040

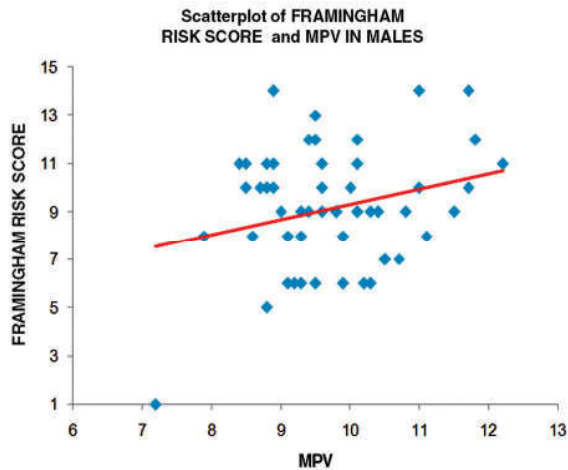**Scatterplot of FRAMINGHAM RISK SCORE and MPV IN MALES**

Fig. There is a significant correlation between MPV and Framingham Risk Score in the males.

Mean platelet volume in females

Mean MPV is 10.00

Range of MPV is 8.3 – 12.1

Correlation of mean platelet volume and the framingham risk score in females.
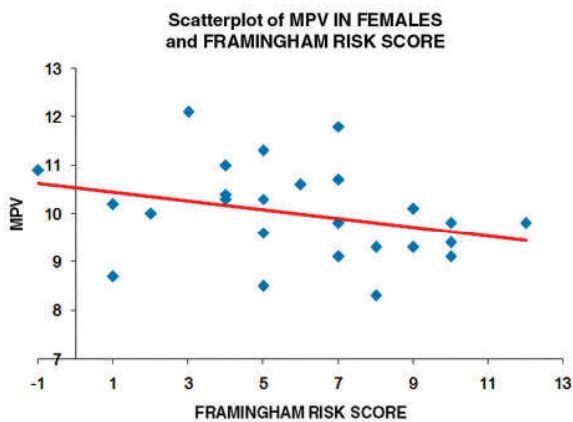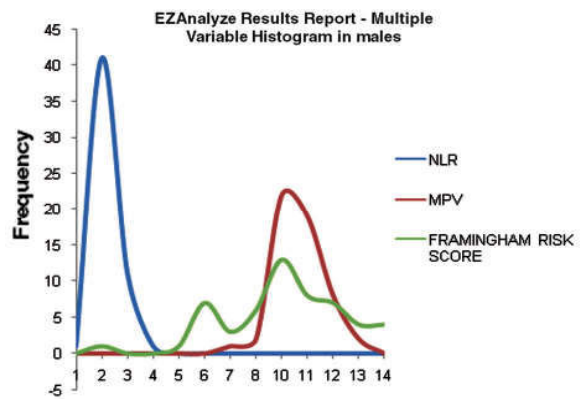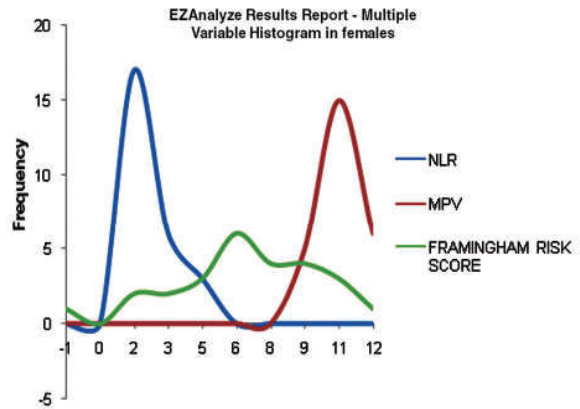
Pearson Correlation	-0.303
p value	0.132

Scatterplot of MPV IN FEMALES and FRAMINGHAM RISK SCORE

Fig. There is no significant correlation between MPV and Framingham risk score in females.

EZAnalyze Results Report - Multiple Variable Histogram in females

EZAnalyze Results Report - Multiple Variable Histogram in males

Discussion

The aim of my study is to find whether NLR and MPV can be used as biomarkers for assessing CAD risk using Framingham risk score among apparently normal individuals. Hence, a cross sectional study with 80 normal individuals was conducted in the Department of Pathology and the Department of Biochemistry in our institution (Govt. Kilpauk Medical College, Chennai, T .N).

The exclusion criteria included individuals with any known comorbidities like diabetes, hypertension, any known infections, smoking, etc.

Normal males aged within 33 to 58 and females aged between 32 to 57 were studied. The mean value of NLR in males was found to be 1.54.Normal range of NLR in males was found to be between 0.79 to 3 similar to that found by Patrice Forget et al.(46) and in females the mean was 1.762 and the normal value is found to be between 0.92 to 4.12.

The mean value of MPV in males was observed to be 9.763 and the normal range of MPV is between 7.2 to 12.2 whereas in females it was observed to be 10 and the normal range between 8.3 to 12.1.

The correlation between Mean Platelet Volume and Framingham risk score among males was found to be statistically significant though it wasn't statistically significant in females. This could have been because the sample size for females was less.

The increased MPV levels are debatable as the risk factor for coronary artery disease as stated by Halbmeyer et al.[68]

Papanas N. et al., Nadar S. et al., Pathansali et al. in their studies reported that MPV has no correlation with known ischemic heart disease risk factors like diabetes, hypertension, and hypercholestrolemia.[69-71]

Study by Sinem N et al has observed that chronic diseases singly did not cause a significant increase in MPV levels. Rather as the number of chronic diseases increase, inflammatory processes underlying the increased cardiovascular risk becomes stronger, and it may affect the platelet activity in favor of atherosclerosis.[72]

There was no significant correlation observed between Framingham risk score and NLR as such although NLR showed an increase with the increased Framingham risk score in females.

Individuals were not categorized into high, intermediate or low risk categories of Framingham risk score in this study. In a study by Neeraj Shah et al. NLR has been shown to reclassify the intermediate risk category of FRS alone.But NLR has been shown as an independent biomarker for assessing risk for CAD.[73]

Contrary to this in a study by Eugenia Quioros et al. the area under the ROC curve showed only a modest, statistically no significant increase with addition of NLR to Framingham risk score.[74]

Erturk M et al. in his study stated that NLR is a cheap and easy to use marker in the diagnosis and the prognosis of CAD patients but definite cut off values are needed to use these hematological markers.[75]

Jun-Bean Park et al. have stated that as none of the inflammatory markers have been established as causative agents, at least at this point in time, it is plausible that NLR, as do all other inflammatory markers, is just a marker reflecting the inflammatory process in patients with CVD, not a key element of the causal chain leading to CVD.[76]

Conclusion

- Neutrophil Lymphocyte Ratio increases with increased risk in females but it was not correlating with the Framingham risk score.

- Thus it cannot be used as a biomarker to calculate the severity of risk of CAD
- Mean Platelet Volume can be used as a low cost laboratory technique for assessing the risk for developing CAD in males as there was a positive correlation between MPV and Framingham risk score
- In females there wasn't a statistically significant correlation with Framingham score maybe because of the smaller sample size compared to males.

Scope and Limitations
- Small sample size of females could have been a limitation
- Patients were not categorized into low, medium and high risk category of Framingham in my study which could have been another limitation.

Summary

The purpose of this study was to find whether NLR and MPV can be used as biomarkers for predicting CAD in normal individuals without any known comorbidities.

Hence a cross-sectional study which includes 80 normal healthy volunteers was conducted in the Department of Pathology and Department of Biochemistry in our institution.

There was a correlation between Framingham risk score and MPV in males but there was no correlation between Framingham risk score and NLR though it shows an increasing trend with increased risk in females .

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