CHAPTER- 6
SUMMARY AND CONCLUSION

In the present study of “Dermatoglyphics and molecular cytogenetic characterization of varicose veins” two hundred and sixteen patients of varicose veins were studied from Pt. BD Sharma University of Heath Sciences, Rohtak, Haryana. The information on the study population was collected by self-administered questionnaire. Diagnosis of varicose veins was done with the help of clinician and categorized on the basis of definition given in the questionnaire. Patients visited the out-patient unit of PGIMS Rohtak and were examined by a surgeon. Patients of varicose veins were subjected to detailed analysis regarding age of diagnosis, sex ratio, clinical symptoms, lifestyle, occupation and molecular cytogenetic detection of FOXC2 mutation. The findings of the study are summarized as follows:

Two hundred and sixteen patients of varicose veins were selected for dermatoglyphic and molecular cytogenetic characterization. The retrospective analysis revealed that out of 216 cases 60.64 % cases were diagnosed between age of 20-40 years. There were 17.12 % who showed varicose veins between the ages of 41 year to 50 years. There were 13.88% of cases between age group of 51-60 years and only 3.70 % cases were having varicose veins after age of 60 years.

There were 127 males and 89 females patients of varicose veins. Out of 89 females there were 24 female who have diagnosed varicose veins at the time of pregnancy. ANOVA on the sample population of varicose veins in different age groups and gender was applied and the interaction of age and gender as factors was studied. Statistical calculation revealed highly significant values for male and female ratio CI = 95%, (df =1 p= 0.0001). Whereas age alone as a factor was found less significant (CI = 95%, df = 4, p= 0.0002). The interaction of gender and age revealed significant values at p<0.0024 (CI = 95%, df = 4).

Varicose veins are classified into four types i.e. saphenous veins, segmented veins, reticular veins and web type veins. Saphenous type of vein was present in eighty two percent of cases. Thirty eight point two percent patients were having segment type of veins. Percentage
frequency of reticular type vein was 28.16% and webbed types of veins were present only in 14% of patients.

Clinical information highlighted seven major symptoms in the present study i.e Prominent veins, aching, swollen ankles, itching, night cramps and ulcer. Prominent veins and aching were observed in 95.08% and 85.24% of patients respectively. There were 31.53% patients with ulcer in one or both limbs and 68.70% of varicose patients complained of night cramps in affected veins. Chi square analysis revealed significant difference between frequency of symptoms among male and female patients ($\chi^2= 16.221^*$, df= 6, p value= 0.01).

On the basis of profession and working the varicose veins patients were divided in three groups. The group I consisted of office workers or those who do less work or live in same position for long time; group II consisted of light physical laborers mainly including arm and leg movement, no weight lifting and no whole body movements; group III consisted of heavier physical laborers including walking, whole body movement and heavy load handling. Out of these, group III workers were most affected (56.48%), followed by group II with 30.98% and Group I worker with 13.27% varicose veins. According to manifestation of symptoms in male and female patients, male patients showed significant association between symptoms and professions ($\chi^2= 22.494^*$, df= 12, p value= 0.03). But in females no significant association was found between symptoms and profession ($\chi^2=16.375$, df= 12, p value= 0.17). Leg ulcer as complicated manifestation of varicose veins was more frequent in patients of heavy load workers in both sexes (In male 79.3% and in female 65%). Chi Square analysis revealed significant association between working groups and sex ratio in varicose veins patients ($\chi^2= 9.629$, df= 2, p value= 0.0081)

Forty two percent patients showed variable complications associated with varicose veins symptoms. Leg ulceration, hematoma and deep vein thrombosis (DVT) were present in 31.53%, 29.30% and 27.77% of cases respectively. Hematomas along with DVT were found simultaneously in 24.46% of cases. There were 16% of patients that showed recurrence of varicosity after surgery. Bleeding from superficial veins was noticed in 10% of varicose patients. Only 02.60 % of patients were seen with femoral vein injury as associated complication of varicose veins.
There were 63.93% of cases who had varicose veins in left limb and 53.11% in right limb. Both limbs were affected in 32%. There were 45.3% men with clinical varicose veins along with symptoms in both legs, 33.8% in the left and 20.9% in the right leg. Women had bilateral symptoms in 14.6%. 51.6% in left leg 33.8% in the right leg. Fisher’s exact analysis showed the two tailed P value is less than 0.00001 and revealed extremely statistically significant association between both sex and limb involvement.

Present study revealed that 37% urban population and 63% rural population is affected with varicose veins. Two way analysis of variation between various age groups and urban/rural population of varicose veins was carried out. The interaction of age and urban/rural revealed highly significant values (p<0.001***, CI = 95%).

Considerable correlation between various risk factors and occurrence of varicose veins was detected in present study. Major risk factors like occupation, working groups, number of pregnancies (in female patients) and obesity were considered. 61.01% patients have reported standing position and 34.24% sitting position at their work. On the basis of occupation percentage frequency of varicose veins in farmer, laborer, electrician, machine turner and fruit seller was 19%, 11.60%, 10.18%, 9.72% and 9.25% respectively. The odds ratio analysis revealed most contributing occupational risk factors as standing posture, indoor work and low temperature at work place.

Pregnancy was found as highly significant factor in female patients of varicose veins. There were 16.27% nullipare, 14.60% primipare, 19.10% secundipare, and 50.56% with three or more pregnancies. Chi square analysis, revealed highly significant values when parity was compared with occurrence of varicose veins.

Obesity and BMI (Body mass index) were also observed as significant risk factor in patients of varicose veins. In patients the average height was 208.87 ± 18.6 cm (limits, 140 to 195 cm), weight was 87.83 ± 17.98 kg (limits, 35 to 115 kg), body mass index averaged 30.03 ± 4.82 kg/m2 (limits, 16.3 to 38.4 kg/cm²). Weight and total BMI showed significant values when analysed statistically (P- 0.0001 Student unpaired t test).

Dermatoglyphic analysis revealed frequency of Ulnar loop as 46% and 59.84% in normal individuals and patients respectively. Whorl pattern was present in 51.9% patients of
varicose veins and in 28% normal individuals. Simple arch and tented arch were present in 02.94% and 07.91% of patients respectively. Normal individuals had 02.86% and 05.41% of simple and tented arch respectively. Radial loop pattern was observed in 03.66% of patients and 02.28% in normal individuals. χ² value revealed the significant variation in fingerprints patterns (For Ulnar loop p>0.008,df-4; Whorl p>0.001,df-4).

Atd angle >65 and <30 was not observed in right or left palm of varicose veins patients. In normal individuals also angle < 30 and > 65 was not found in left palm and there were only 0.4% individuals with angle between 60-65 in right palm. Atd angle of right hand of varicose veins patients and control group (normal individuals) showed non significant values at 5% level. t’ test analysis revealed non significant values between frequency of sum of total atd angles (right +left) of varicose veins patients and control group, p=0.8233 and p= 0.7079 for right and left palm respectively.

In the present study, out of 216 varicose veins cases, 190 cases were subjected to chromosomal investigation. Cytogenetic investigations using trypsination and G banding revealed normal karyotype. Patients who had severe varicose veins with ulcer and other complications showed normal karyotype. Similarly no chromosomal anomaly could be detected in patients with history of recurrence of varicose veins.

Patients of varicose veins were subjected to molecular cytogenetic analysis to identify the mutation in FOXC2 locus. Out of 216 patients of varicose veins, 190 patients were analyzed for mutation detection. Allele specific PCR was done for the detection of mutation in FOXC2 gene in region I.

DNA was isolated by optimization of the protocol which resulted in 80ng to 100ng of DNA. Purity of DNA sample was checked at the OD 260/OD 280 for reference ratio of 1.65 to 1.85. The samples which were found to fluctuate from the reference range were purified again by RNAse and Protienase K treatment. The purified DNA was stored in the TE buffer (pH 7.6) at -20°C.

Purified DNA was amplified using polymerised chain reactions. Amplification was carried out to amplify -413 bp of the 5’ regions to +655bp of exon 1 of FOXC2 gene. Due to the presence of a high G + C content in the target DNA template, in vitro DNA amplification was
done by improvisation. Allele specific PCR was used with certain modifications like adding enhancers (DMSO and glycerol) in the reaction buffer to get the amplification. To study genetic variation among varicose veins patients and normal individuals, 655 bp of single exon of FOXC2 gene as well as additional 413 bp of upstream 5’ UTR was sequenced for the confirmation of SNPs involved.

Three SNPs -91C→G, -41G→A and -41G→T were detected in the patients of varicose veins. Out of 190 patients of varicose veins only 32 were detected with -91C→G mutation of this fragment. Among 32 patients who showed this mutation number of male patients was 24 and females were 8. 122 patients were detected with -41G→A mutation. Out of 122 patients there were 83 males and 39 females who showed this mutation. Hundred patients were detected with -41G→T FOXC2 mutation of fragment 450 bp. Out of which there were 48 males and 52 females’ patients of varicose veins. One hundred two male patients and sixty seven female patients were showed at least one mutation in region I of FOXC2 gene. $\chi^2$ value revealed a significant association between types of mutation and sex ratio of varicose veins cases (df = 2, p>.002). FOXC2 mutation analysis in region I showed that frequency of mutation was 57% in heavy work load laborers and 31% in light physical labors. Office workers were having mutations in 12% cases. Fisher’s exact analysis revealed a significant association between three working groups, sex ratio and FOXC2 mutations (p< 0.05). $\chi^2$ analysis between types of mutation and professional groups ( group I,II and III) of varicose veins cases also revealed significant association ( $\chi^2$ = 16.021, df = 4 and p>.002). Odds ratio analysis revealed that group I was less likely affected with FOXC2 mutation than group II and group III.

DNA of 78 severe cases (having more than two critical symptoms of varicose veins) was subjected to DNA sequencing for detecting the mutation in another locus of FOXC2 gene. Sequence analysis of 78 patients revealed four mutation in 69 patients in the region II of FOXC2 gene, of which there were two missense (c.1205C>T and c.1331A>G) and two frameshift mutations (c.902-920dup19 and c.876-877delCG). Out of 78 selected patients there were 52 males and 26 females. Forty eight males and twenty one females showed mutation in FOXC2 gene. 78% of males who showed mutations were working as of heavier physical laborers including walking, whole body movement and heavy load handling. Remaining 22 % males were light physical laborers, office workers or those who do less work did not show these mutation.
Out of 21 women with mutation, 15 (73%) were those who had three or more pregnancies, 19% were secundipare and remaining 8% were nullipare.

In present study it was found that the occurrence of varicose veins was more frequent in patients with a positive family history mainly in the case of father affection. One hundred thirty two patients had family history of close relatives suffering from varicose veins. Family history screening revealed 63.63% cases of development of varicose veins, when both parents were affected, whereas the risk was only 19% for individual who had unaffected parents. Out of 132 patients with family history of varicose veins, only twelve patients had at least one person from their family affected with varicose veins. Sixty eight patients were those who had two affected person in their family members and fifty three were with three or more affected person. According to prevalence, family history indicated a high risk of varicose veins. Age and body mass index adjusted OR was 1.7920 (95% CI: .5119-6.273). The prevalence of varicose veins in those with positive family history was 61.11%, 39.81% in men and 21.29% in women. Adjusted OR was 6.375 (95% CI: 0.7508-54.126) in men and 0.319 (95% CI: 0.0319-3.181) in women.

Out of 132 patients with family history of varicose veins 72 families have given consent for mutation detection and pedigree analysis. Presence and absence of mutation was checked in all family members. Mutation analysis of these cases revealed that out of total 72 mothers, 51 were found with at least one FOXC2 mutation. Father of 63 patients have been detected with mutations. Out of 46 grandparents, 19 grandfather and 14 grandmothers have shown the mutation in FOXC2 gene. Siblings of 24 patients were found with FOXC2 mutation and had the varicose veins symptoms whereas rest of siblings were normal.

Present study revealed that out of seventy two families, twelve families had both affected parents and 70% affected offspring. Genetic mutation in FOXC2 locus was detected in these patients. In present study it was found that out of 72 studied families there were eleven families who had varicose veins in all three generations with at least one FOXC2 mutation. This strongly suggests genetic predisposition and involvement of FOXC2 locus in etiopathogenesis of varicose veins. Evaluation of patients of varicose veins and their family members have highlighted strong genetic predisposition in occurrence of varicose veins. Role of various risk factors as well as mutation in FOXC2 locus have been depicted as well as validated in the present study.
Screening of parents and family members revealed that history of varicose veins in first degree relatives was the most important cause for varicose veins in both sexes. Along with family history, environmental change, lifestyle, pathological and gene mutation led to physiological alteration in vein system. All these factors may have contributed to venous insufficiency and finally varicose veins formation.

Determining a specific etiologic diagnosis is central to understand the nature of the problem. It is very helpful in providing answers to questions regarding prognosis, recurrence risks, directing specific therapies, and achieving meaningful inclusion of individuals with disease. A diagnosis of FOXC2 gene will be helpful in early treatment and intervention strategies which can contribute to improvement in outcome. To fully understand the genetic contribution to VV disease, there is a need to formulate a rigorous research protocol which should utilise some of the very advanced genetic approaches. Further analysis of FOXC2 gene should be done to detect the presence of other SNPs and to define other haplotypes. Genetic profiling of varicose veins will be helpful in early detection of disease and accordingly measures to prevent the disease would be taken.

THE MAJOR OUTCOMES OF THE STUDY:

- Prevalence of varicose veins had been studied in the state of Haryana. Present study is first of its kind to establish the role of risk factors in occurrence of varicose veins. Present study points out the role of age, sex ratio, urban/rural, life style and environmental factors in the occurrence of varicose veins in the population of Haryana. Prevalence of varicose veins was noticed maximum in 20-40 years of age which indicated that a new varicose vein could also occur before middle age.

- Sex ratio analysis pointed towards more number of males suffering from varicose veins.

- Overweight and height seemed to be significant risk factors of varicose veins. Therefore among varicose veins patients, body mass index may be associated with an increased risk of varicose veins. Considerable effects of sex, age, overweight and pregnancy was noticed on the occurrence of varicose veins.
Presence of more frequency of affected person in rural population and more patients in working area of low temperature is highlighting the role of environmental factors in etiology of varicose veins.

Standing at their work place for long time was significant risk factor of varicose veins. Heavy load workers (group III) were having maximum cases of varicose veins. These all occupation and life style of an individual have a major impact on the occurrence of varicose veins.

Certain associated complications like ulcer, bleeding, deep vein thrombosis, hematoma and recurrence have been found as a serious indicator of varicose veins. Majority of patients presented with complications of varicose veins rather than the disease itself also.

Cytogenetic analysis revealed no gross numerical and structural chromosomal anomalies in patients of varicose veins as well as in severe cases of disease. Therefore cytogenetic analysis is not required in the patients of varicose veins. However, less than 500 bp structural anomalies could be present in the patients of varicose veins. Hence the study supports the need of molecular examination in cases of suspected patients of varicose veins.

Foxc2 mutation either region one or region two was found in all most all varicose patients. Thereby reflecting the strong genetic component as a causative factor in varicose veins.

Parental mutation detection and pedigree analysis had confirmed the heritability of varicose veins and thereby suggested a notable genetic component in the etiology of the disease.

Early diagnosis of varicose veins disease will certainly influence treatment and intervention strategies. This will further lead to positive outcome and worsening of disease would be prevented.

SIGNIFICANCE
Present study is first of its kind to establish the role of risk factors in occurrence of varicose veins. Role of various risk factors as well as mutation in FOXC2 locus have been depicted as well as validated in the present study. Symptoms in early varicose veins are minimal and they become severe with time. One must know early symptoms and diagnosis should be made early to avoid painful and dangerous complications. The present information will be a great help to the clinician to evaluate and diagnose the varicose vein disorder in early stage. Eventually symptoms and suffering from the disease can be prevented by early intervention and taking precaution as per advice of clinician.

GUIDELINES:

- In each patient, clinical history should be taken irrespective of degree or severity of varicose veins. Information regarding number of pregnancy in females, lifestyle and working environment is mandatory to find out probable risk factor of varicose veins.

- Varicose veins should be diagnosed and assessment should be done by detailed clinical and morphological examination as well as Doppler ultrasound as these will remain the basis of etiological diagnosis.

- Molecular cytogenetic analysis with the help of allele specific primers to detect mutation in FOXC2 locus is strongly recommended in patients of varicose veins. Early diagnosis with intervention will certainly minimize VV complications for the patient. This will be helpful for genetic counseling also.

- As venous insufficiency can predispose to thrombosis, it is suggested that all patients should be screened that FOXC2 mutations for early detection of disease.

Varicose veins of lower extremities are a very frequent problem that affects most of the men and women and in some cases child below age 15 too. It is one of the common disease, mostly ignored by the people. Due to negligence by people and ignorance of varicose vein it ends up in long lasting leg ulcer. The list of occupational hazards is increasing day by day. Varicose veins have become a serious problem of millions of people across the globe and ignored by people living across India. Many people suffer from it, but most tend to ignore it and that is not good as it can lead to complications in the advanced stage. In India there is a lack of
education on this subject as the common man doesn’t know what the problem is all about and what can happen if it is not treated on time. So awareness programme regarding of varicose veins will certainly be very helpful in preventing the disease.

FUTURE PROSPECTS:

- To fully understand the genetic contribution to VV disease, there is a need to formulate a rigorous research protocol which should utilise some of the very advanced genetic approaches. There are several approaches available to determine the genetic link. **Gene expression profiling using microarray technique** can be used in detail analysis of varicose veins. **Candidate gene studies** have shown the involvement of **Notch3 gene mutation, desmuslin, thrombomodulin and methylenetetrahydrofolate reductase** with varicose veins analysis of more genes is an open area.

- Though there is a high prevalence of varicose vein in India, very few studies have been conducted in India. Peoples are under the risk of development of varicose vein since they are forced to stand for long time for their work. Researcher personally has observed that most of the Indians were unaware of varicose veins, and also their preventive measures. So there is a **need to educate the people** regarding this condition in order to prevent it. Data on prevalence and on attributable risk factors are important for the research on the prevention of varicose veins. It is highlighted in the present study that both environmental and genetic factors are associated with the development of varicose veins. The present study would help in the early detection of associated risk factors of varicose veins which would be quite helpful to reduce the risk of the disease.

- Understanding the pathological pathways underlying unexplained forms of varicose veins represent a future challenge to increase both prevention and possible therapies. Presence of FOXC2 mutation reflects the strong genetic component as a causative factor in varicose veins. Molecular analysis will be helpful in finding the gene level mutation and this will be confirm the underlying cause behind varicose veins in population and help as prognostic marker and predisposing factor for varicose veins. As it is known that variation in metabolic profile is stably controlled by genetic and environmental factors therefore by introducing a **case–control study methodology**, disease phenotypes and
genetics component can be directly correlated. This will further lead to establishment of diagnostic biomarkers.

- Synchronised use of technologies including genome-wide association study (GWAS), metabonomics (Chemical process involving metabolites) and micro-RNA analysis have the potential to elucidate the molecular mechanism of the disease and to identify the prognostic marker of varicose veins. The implementation of the new technologies will allow the analysis of whole-genomes, which could lead to detect single base mutations and structural variations involved in occurrence of varicose veins.

- The causes of phenotypic variability need to be explored in genetically linked venous syndromes. Finding out the prevalence rates of these syndromes in different sections of society and well-defined specific clinical diagnostic criterias will give an insight regarding prognosis, risks, therapies and preventive measures. This will be of remarkable help to reduce the occurrence of varicose veins.

- There is clearly a genetic component to venous disease leading to histologic and molecular alterations. The degree of clinical severity is compounded by lifestyle and other demographic factors on top of this. Genetic screening and gene-targeted therapy could eventually play a role in the clinical management of varicose veins in the future and will helpful to eradicate chronic venous ulceration.