Chapter 5

Summary and Conclusion
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*Genetics seemed to promise incredible advances in the fight against disease, yet new cures and treatments have been slow to arrive. Why?....*

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Multifactorial diseases, such as cardiovascular diseases, can be as complex as their name suggests. Cardiovascular diseases are on rise worldwide, and they are currently the leading cause of mortality in developing nations. This disease is partially explained by genetics, and it is known to run in families (Watkins & Farrall, 2006). However, lifestyle and environmental factors, such as diet, also contribute to coronary artery disease and heart failure. Thus, this disorder shows multifactorial inheritance, because both genetic and environmental factors trigger its onset. Present study attempted to understand, estimate and document the distribution of various cardiovascular diseases and associated risk factors and their interaction with methylenetetrahydrofolate reductase (MTHFR) gene among the Jat community of Haryana. As aforementioned, adverse cardiovascular phenotype is interplay of gene and environment, the study also attempted to look into the gene (MTHFR C677T)-environment (smoking, alcoholism, lipids, and nutrients-folate and vitamin B12) resulting in hypertension and metabolic syndrome. Moreover, reduced MTHFR activity of this gene results in increased homocysteine levels (hyperhomocysteinemia) that can further lead to other cardiovascular adversities. The study was conducted in Palwal district of Haryana covering three different blocks and a total 14 villages where Jats were predominantly settled. The study involved two phases namely pilot survey and field work. Prior to the pilot survey, necessary interview schedules were formulated and ethical clearance (Annexure I) was also obtained from the ethical committee, Department of Anthropology, University of Delhi, Delhi. A pilot survey was conducted in the month of July, 2011 to establish a good rapport and to have feasibility value to carry out research work in the selected populations. The interview schedules including the questions involving anthropological and clinical aspects were pre-tested from each
population and were modified later. Primary data pertaining to life style (through interview schedules), anthropometric, and physiological parameters (through measurements using standard techniques) were collected. Five ml of intra venous blood was collected in EDTA coated vacutainer (qualified and authorized person was deployed for the purpose) using disposable single use syringes with prior informed written consent (attached as Annexure I) utmost care was taken to avoid relatives upto first cousin. Collected samples were further used for biochemical and molecular analyses. Anthropometric and physiological measurements undertaken during present study were collected using the standard methods. Ethical clearance was obtained from departmental ethical committee.

In the present study the data was collected from 1387 individuals belonging to distribution in 15 villages of palwal district Haryana India. Clinical data was based on the medical details of the subjects from already available reports with them. A biochemical analysis was done in All India Institute of Medical Sciences (AIIMS), Delhi. Collected blood samples for molecular analysis were stored in ice box and transported to the Molecular Anthropology Laboratory, Department of Anthropology, University of Delhi. Samples were subjected to Deoxyribonucleic acid (DNA) extraction within one week of collection. Extraction of DNA and further molecular analysis were done in the Molecular Anthropology Laboratory, Department of Anthropology, University of Delhi. Extraction of DNA of the collected blood samples was done according to Miller et al. (1988) protocol. Isolated DNA from white blood cells (WBCs) were re-suspended in Tris EDTA (TE) buffer and made dissolved by keeping at 370C overnight. Dissolved DNA samples were quantified using spectrophotometer Nanodrop and then preserved in -800C for further molecular analysis. All the samples were subjected to Polymerase Chain Reaction (PCR) for MTHFR C677T polymorphism detection in the Molecular Anthropology Laboratory of Department of Anthropology, University of Delhi following the standard protocol. Appropriate statistical softwares were used to analyze the generated data.

In the present study, the median age of the population was found to be 46 years. Males (50yrs) were significantly older than females (45yrs). The studied Jat population comprises of more females (69.00%) than the males (31.00%). Literacy rate in the
present study was found to be 45%. Major occupation was found to be agriculture. Almost all individuals (99.04%) participating in the present study are found to follow vegetarianism and consumption of milk and milk products in the form of curd and lassi was found to be a daily routine. Smoking (45.71%) and the use of tobacco (in the form of traditional hukkahs and bidis is found to be very common among Jats of Haryana. It is noteworthy that females of this community also smoke; although more number of males were found to be smokers.

All the subjects were screened for the prevalence of complex cardiovascular disorders. Among the observed complex disorders, CAD (0.50%) and T2DM (0.36%) were found to be very less frequent among the studied population. No incidence of stroke was observed. 10% of the individuals were found to have established hypertension; further, 28% were identified to have hypertension and 37.8% as having pre-hypertension during the field work. Of the total 1387 individuals, only 210 individuals were not found to have any of the above mentioned complex phenotypes. In other words, one can say that approximately 80% individuals were having one or the other adverse cardiovascular phenotype. Moreover, individuals diagnosed with T2DM and CAD were found to be in very less frequency. The total individuals 3.82% were found to have multiple complex disorders (individuals having more than one complex disorder at a time). Males were found to have comparatively higher prevalence of all the adverse cardiovascular phenotypes than the female. *This low prevalence of the disease among Jats of Haryana infers that this group is likely to be in the first stage of epidemiological transition.*

Median levels of majority of the parameters fell in normal range. Traditional (WC+WHR) and non traditional (BF%, FM, FFM) measures of obesity are above the normal range being significantly higher among females than males. However, biochemical and physiological factors were higher among males than the females. Individuals with abnormal levels of majority of the variables were found to be quite frequent in the Jat population. More than 10% of the individuals have some or other risk factor. WHR and WC showed the highest prevalence among the traditional risk factors. The prevalence was in the order - high abdominal obesity (WC & WHR)>hypertension>generalized obesity (BMI)>dyslipidemia in the order-hypercholesterolemia>high HDL>hypertriglyceridemia>low HDL>high blood glucose.
Among the non-traditional risk factors, the prevalence is in the order: hyperhomocysteinemia > high body fat > low vitamin B12 > high non HDL > pre-hypertension > low lean mass > high fat mass > MS > low folate > high ETEW (HTGW). Men have majority of the risk factors abnormal as compared to women except for measures of obesity that are higher among women. Presence of high number of adverse variables in this population is indicative of the adverse effects that this population may have in their later lives. It is noteworthy that body fat percent explained the ‘normal weight obesity’ existing in underweight and normal weight individuals as classified by BMI. 34% of the underweight and 47% of the normal weight individuals had high body fat. This shows that BMI alone may not be a true indicator of obese profile among the Jats of Haryana and body fat percentage might be a better marker and predictor of fat composition and risk obese profile.

Growing evidences suggest that pre-hypertension, a pre-stage to hypertension is equally important when the risk for complex diseases like CVD is considered. Therefore, these two phenotypes were further dealt with in detail considering them to be complex phenotypes as cardiovascular outcomes. The hypertensive individuals were the oldest (median age: 52yrs), followed by pre-hypertensives (median age: 46yrs) and normotensives (median age: 45yrs). Prevalence of pre-hypertension and hypertension was found to be 37.8% (39.59% among men and 38.67% among women) and 38.0% (46.15% among men and 36.29% among women) respectively. Smokers and alcoholics were found to be significantly higher in pre-hypertensives and hypertensives than the normotensives. Male gender was significantly associated with pre-hypertension and hypertension showing that men are at higher risk for both as compared to women. The present study showed that as compared to normotension, prehypertension and hypertension are associated with an increased prevalence of other CVD risk factors. Central obesity was found to be a major determinant of both pre-hypertension and hypertension even after controlling for age and other probable confounders. Major characters of obesity identified in this population were found to be HTGW and BF% that were found to be important contributors of both pre-hypertension and hypertension. It is noteworthy that obesity in terms of BMI, HTGW, and BF% was found to be significantly associated with hypertension and that in terms of HTGW and BF% was
found to be significantly associated with pre-hypertension. BMI is a measure of height and weight which is more genetic while WC is more of an environmental factor and therefore more likely modifiable. HTGW is calculated by hypertriglyceridermia and high WC both of which can be modified by life-style modifications. Present study adds to the emerging evidence on the use of hypertriglyceridermic waist to identify individuals characterized by the vascular risk. Association of BF% with hypertension in the present study is another noteworthy and significant result. Studies say that the BF% estimation allows for more precise analysis of body components than simply BMI and WC measures. Through BF%, lean body mass and fat mass can be estimated. In this sense, if equations that estimate BF have good predictive capacity for hypertension, they can, while analyzing body composition, identify people at risk for hypertension (REF FROM Silva et al., 2012). Current guidelines recommend that lifestyle modifications can lower the risk of pre-hypertension. Moreover, prehypertension is a characteristic of young ages while hypertension is that of older ages. Early diagnosis of pre-hypertension and its treatment during the pre-clinical stages can actually lower the incidence of hypertension in older ages, thereby, reducing the risk of overt hypertension.

Population was further screened for the presence of metabolic syndrome (MS). Of the total 1234 individuals for which metabolic syndrome was screened, metabolic syndrome was present among 26% as measured by NCEP-ATPII criteria and 23% as measured by IDF criteria. In comparison to individuals without metabolic syndrome, those having metabolic syndrome were older, taller and heavier and smoking was found be more common. Relatively more number of females have MS than the males. Most of the variables were found to be significantly higher in individuals with MS than those without it. Further, distribution of abnormal CV variables also showed similar trend. As depicted by the median values, individuals with abnormal levels of all variables except folate, were found to be significantly higher among MS cases as compared to those without MS. This shows that abnormal levels of parameters that are not included in the definition of MS i.e. homocysteine, vitamin B12, non-HDL-C, HTGW, and BF% are also associated with MS in this population. In the present study, the central components that have been identified are pre-hypertension, HTGW, BF%, BMI, WHR and non-
HDL-C in addition to established risk factors included in the characterization of MS as identified by the multivariate analysis. This highlights the risk that these parameters pose for MS, thereby further predisposing these individuals to the risk of complex diseases like CAD. *Traditional risk factors combined with MS results in increased global cardiometabolic risk. This high prevalence of cardiometabolic risk in the present population hints towards the shifting of this population from first stage towards the third stage of epidemiological transition as against inferred by the low prevalence of CVD particularly CAD, T2DM and stroke.*

Further work in the present study included the screening of MTHFR C677T polymorphism in this population and to assess its relation with studied cardiovascular variables and outcomes as this polymorphism has been implicated in various diseases like CVD. C677T polymorphism was analyzed in 1187 individuals and this SNP was found to be polymorphic in the present population. The population was found to be in Hardy-Weinberg Equilibrium with respect to MTHFR C677T polymorphism. The mutant T allele frequency of MTHFR C677T is found to be higher among males (16%) than the females (15%) but the difference is not found to be statistically significant.

Age wise distribution of this polymorphism revealed no significant difference between the age groups ≥50 years and < 50 years with respect to MTHFR gene polymorphism. Lower frequency of T allele among the younger group is indicative of a selective disadvantage of the allele in the population in recent years.

Further analysis showed that individuals carrying ‘T’ allele are comparatively older than those carrying ‘C’ allele. No gender differences were observed with respect to the genotypes. Individuals with TT genotypes were more obese in terms of WC, supra-iliac skinfold and BF% (borderline significance) than those with CC and CT genotypes. Individuals with CT genotype had higher (borderline significance) glucose levels than with ‘CC’ genotype. Individuals with CT & TT genotypes had relatively abnormal lipid profile as compared to individuals with CC genotype; HDL having borderline significant difference. Further, prevalence of cardiovascular risk factors was estimated among individuals carrying ‘C’ allele and those carrying ‘T’ allele and it were found that the latter have higher prevalence of most of the abnormal parameters with high
LDL, high non HDL-C (borderline significance), and low folate showing significant difference.

Association of MTHFR C677T with complex phenotypes/cardiovascular outcomes was further investigated. Frequency of T allele was found to be similar (17%) in both normotensives and hypertensives as against 15% present in pre-hypertensives. No significant risk could be attributed to both pre-hypertension and hypertension with respect to this polymorphism under any of the inheritance models. Further, multivariate analysis adjusting for age and sex also revealed no risk associated. This shows that MTHFR C677T does not have any independent association with either pre-hypertension or hypertension. On applying Kruskal Wallis test, age, WC, WHR, cholesterol, non HDL-C, LDL, and vitamin B12 showed significant association with MTHFR C677T among pre-hypertensive individuals. However, no such association could be observed among hypertensive individuals. This infers to the risk of MTHFR C677T for pre-hypertension in the presence of these variables. Although no such association is observed with hypertension; the indirect risk as can be conferred by pre-hypertension which is further a risk for overt hypertension is noteworthy indeed.

Further association analysis with MS revealed that TT genotype of MTHFR gene C677T polymorphism was significantly higher among the cases as compared to controls. The frequency of mutant ‘T’ allele is found to be relatively higher among MS cases (16%) than the controls (15%) but the difference is not statistically significant. TT genotype under co-dominant and recessive inheritance models showed more than two fold significant increased risks for MS. CT genotype is not found to have any risk associated with MS. This shows that T allele when present in double dose is associated with MS. However, on adjustment for other risk variables, TT genotype shows more than one fold risk for MS; risk being not significant. On applying Kruskal-Wallis test, significant association of TG and VLDL with respect to MTHFR C677T is observed among individuals with MS. LDL and non HDL-C also showed association with MTHFR C677T but with limited significance (borderline significance; p=0.056 and 0.06; respectively). This refers to the risk of MS in T allele carrying individuals in the presence of TG, VLDL, LDL and/or non HDL-C.
Hypertension and metabolic syndrome are complex disorders and their expression is controlled not only by genes but also by interaction of gene with the environment. In prehypertensives, CT genotype showed 3 fold significant increased risk with alcoholism status. This infers to the risk of prehypertension in individuals who consume alcohol and also have CT genotype. In hypertensives, significant interaction of CT genotype with low HDL shows the risk of this genotype in the presence of low HDL. In individuals with MS, CT showed significant interaction with TG, VLDL, HDL, non HDL and TT genotype showed significant interaction with glucose, TC, LDL and folate in addition to the aforementioned variables. This infers towards the risk of these genotypes for MS in the presence of altered levels of these variables.

MTHFR C677T may not be directly involved in the pathogenesis of pre-hypertension, hypertension and metabolic syndrome but its interaction with the environmental variables increases the risk for the development of these adverse phenotypes. Hence, lifestyle modifications such as smoking and alcohol cessation and screening programs for nutritional deficiencies in various ethnic/community groups followed by counseling and supplementation programs may help reduce the burden of the complex diseases.

Further, in the present study, future 10 year risk of adverse cardiovascular events was assessed in pre-hypertensives, hypertensives and metabolic syndrome individuals inorder to understand the utility of these phenotypes in pre-disposition to later life cardiovascular events. approximately, 9.5% of the total individuals had risk score above 10%. On assessment of risk score among individuals with pre-hypertension, hypertension and metabolic syndrome and comparing them with their respective controls, it could be observed that the individuals with the complex phenotypes have relatively higher risk score than their respective controls. Pre-hypertensive and hypertensive individuals have twice and thrice respectively greater risk of developing future CVD in near 10 years than the normotensive individuals. Individuals with metabolic syndrome are also at twice greater risk of developing future CVD in near 10 years. 7.5% of the women and 13.75% of the men are at a risk of developing future CVD. Men have more than twice greater risk of developing future CHD in near 10 years as compared to women.
Further, the assessment of the risk of MTHFR C677T for future CVD events, MTHFR was not associated with future 10yr CVD risk; but is associated through smoking, alcohol consumption, HDL and homocysteine. It is noteworthy that MTHFR not associated with hyphcy as seen in previous analysis, but it seems that it has an adverse effect in hyperhomocysteinemic individuals increasing future CVD risk. Therefore, study further investigated the reasons of hyperhomocysteinemia in this population (outside the objective of this study). 

A separate computation of hyperhomocysteinemia as a major risk phenotype of CVD events was further carried out in order to unravel the causes of hyperhomocysteinemia (hyphcy) in the present study and to evaluate contribution of the individual variables (MTHFR C677T) in the development of the phenotype (Hcy level), and to get an estimate of the relative contribution of environment (vitamins) in modulating the effect of genotypes in the present population. Older age and low folate and vitamin B12 levels are likely associated with the risk of hyperhomocysteinaemia. Moreover, approx. 20% of the underweight individuals have hyphcy and show an increased risk; though the risk is not statistically significant. Underweight has been found to be a risk for hyperhomocysteinemia. However, its risk for hyperhomocysteinaemia in a general population needs further investigation.

Risk for hyperhomocysteinaemia under various nutritional conditions showed that 65.4% of those deficient in both vitamin B12 and folic acid had hypHcy. 56% of those having optimum levels of both showed hypHcy. Incidences of hypHcy in individuals with only B12 or only folic acid deficiency were respectively, 71% and 62%. In subjects with only vitamin B12 deficiency, the relative risk for hypHcy was twice than those with optimum level of both. Folate deficiency alone or in combination with vitamin B12 deficiency showed no significant increased risk for hyperhomocysteinaemia. Lifestyle is the most important environmental factor determining plasma tHcy concentrations. Studies have also investigated the role of smoking and alcohol in increasing homocysteine levels. Smoking in the form of bidis and hukkahs is extensively prevalent among the Jats of Haryana as also shown in chapter 3. Jats of Haryana smoke bidis rather than cigarettes; former is more dangerous than the latter.
Smokers and alcoholics are found to have respectively, 1.5 fold and 4.5 fold risks for hyperhomocysteinemia. Further, smokers and alcoholics show higher incidence of folate and vitamin B12 deficiency than the non-smokers. Extensive smoking and alcoholism may be the factors other than vitamin deficiency associated with hyperhomocysteinemia and may be the reasons for the prevalence of high percentage of hyperhomocysteinemia in this population.

Further gene-environment interaction analysis showed that T allele as such may not be significantly associated with hyperhomocysteinemia. However, in the presence of vitamin B12 deficiency alone or in combination with folate deficiency, T allele results in hyperhomocysteinemia.

Despite the high prevalence of hyperhomocysteinemia in the present population, they show a very low prevalence of major CVD outcomes like CAD and stroke (not found in the present study). HHcy as a potent pro-inflammatory factor might accelerate the development of atherosclerosis. However, hyperhomocysteinemia is not found to be a risk factor for hypertension or metabolic syndrome in the present study. The human immune system works in such a way that whenever there is an increase in pro-inflammatory, anti-inflammatory markers increase to maintain a balance between pro-inflammatory and anti-inflammatory markers that is necessary for normal human immunological functioning. Therefore, the present study further tried to look into this aspect of immunology in order search an answer for the non association of hyperhomocysteinemia with metabolic syndrome and further a low prevalence of existing CVD among Jats of Haryana in the present population via FOXP3 gene expression (beyond the objectives of the present study). Majority of the hyperhomocysteinemic individuals had vitamin B12 deficiency but had normal folate levels. Further, majority of the hyperhomocysteinemic individuals had increased FoxP3 expression. Individuals with normal level of folate, therefore, are likely to have increased Fox P3 expression. Findings of the present study reflects the folate mediated enhanced Fox P3 expression in hyperhomocysteinemic and metabolic syndrome individuals that has halted their progression towards developing more complex phenotype like CVD in the presently studied population.
Thus, if one side of the coin is looked at, based on the low prevalence of CVD and controlled risk of hyperhomocysteinemia through FOXP3 expression, it seems that Haryana is still straddling somewhere in the first stage of epidemiological transition. However, when looked at the other side of the coin, high prevalence of traditional risk factors and emerging non traditional risk factors along with metabolic syndrome and the interaction of MTHFR C677T with them; thereby increasing the cardiometabolic risk (also evident from 10yr future CVD risk score), infers that this group is heading towards the third stage of epidemiological transition likely to the enter the this stage in near future. Therefore, understanding the prevalence of CVD associated risk factors and identifying the role of emerging non traditional risk factors along with the genetic makeup of such population groups who are likely to undergo epidemiological transition urge the need of formulation of interventional strategies that may be useful in combating the forthcoming adversities.