**INTRODUCTION**

Impaired homocysteine metabolism (hyperhomocysteinemia, a pro-thrombotic risk factor) has been extensively studied in different subsets of patients as well as different ethnic groups. Population data reveal a great variation in the distribution of hyperhomocysteinemia, ranging from 0% to 86%, attributed to a polymorphism in methylenetetrahydrofolate reductase gene (MTHFR; rs1801133) and variable folate and/or vitamin B12 levels. Being at the axis of DNA synthesis and methylation cycle, homocysteine metabolism (in close proximity with MTHFR) plays an important role in lipid metabolism, immune activation and other biological reactions. Hypomethylation of homocysteine has been associated with dyslipidemia, atherosclerosis, hypertension, and all cause mortality in different populations of the world. Also, hyperhomocysteinemia is known to suppress the function of Foxp3+ Tregs cells in murine models, suggesting an important link between homocysteine metabolism and Foxp3. Further, interleukin 17 and 10, referred to as pro-atherogenic markers, have been associated with higher levels of homocysteine in different populations.

**Background:** Impaired homocysteine metabolism (IHM; hyperhomocysteinemia) has been linked with many complex disorders like cardiovascular diseases and immunological disturbances. However, studies understanding IHM in light of pro- and anti-atherogenic markers like Interleukin-17A & -10 (IL-17A & IL-10) and Forkhead box p3 (Foxp3, a master transcription factor) are scarce.

**Aim:** In our present study, we aimed to understand the relation of IHM with plasma IL-17A and IL-10 levels and Foxp3 mRNA expression in peripheral blood mononuclear cells (PBMCs) from an endogamous population (Jats of Haryana, North India) with high prevalence of IHM without the concurrence of significant adverse cardiovascular outcomes.

**Methods:** Forty (40) clinically healthy individuals, unrelated up to first cousins, were recruited and were subjected to demographic, physiological and anthropometric profiling, followed by intravenous blood sample collection (fasting) and lipid profiling. Plasma homocysteine levels were estimated and individuals with homocysteine levels ≥ 15umol/L and <15umol/L were categorized as the impaired homocysteine metabolism group (IHM, n=30) and normal homocysteine metabolism group (NHM, n=10) respectively. Plasma folate and vitamin B12 and MTHFR C677T (methylenetetrahydrofolate reductase) polymorphism were detected. Relative mRNA expression of Foxp3 in PBMCs (normalized to 18S) was quantitated using SyBR green technology. Plasma IL-10 & 17 levels were estimated by ELISA assays.

**Results and Conclusion:** None of the physiological, anthropometric and lipid variables were different between the two groups. Foxp3 mRNA expression levels were relatively lower, and plasma IL-10 levels were found to be comparable among IHM and NHM group. However, significantly higher IL-17A levels and relatively high LDL cholesterol levels were present in the IHM group as compared with NHM. Our findings suggest that the Jats of Haryana, North India, exhibiting high levels of homocysteine, might also carry the high IL-17A -pro-atherogenic marker, suggesting an increasing burden of pre-morbid condition. This apparently does not reach to significant mortality/morbidity attributed to the counter action or balancing act of IL-10 (an anti-atherogenic marker). This further suggests environment-influenced epigenetic control mechanisms of the targeted genes in the present population. Ethn Dis. 2018;28(4):525-530; doi:10.18865/ed.28.4.525.

**Keywords:** Homocysteine Metabolism; MTHFR; Foxp3; Interleukin-10; Interleukin-17A; Folate; Vitamin B12

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pro- and anti-atherogenic cytokines, respectively, are also regulated by Foxp3. Previous studies report independent association of hyperhomocysteinemia and interleukins (IL10 & IL17) with complex disorders, but the immunological aspect of homocysteine metabolism through Foxp3 and associated cytokines, in light of folate, vitamin B12 and MTHFR C677T polymorphism, in apparently clinically healthy patients has not been investigated. Recently, we reported high prevalence of IHM and vitamin B12 deficiency, without concurrence of high rates of adverse cardiovascular outcomes, despite significant metabolic distress in terms of dyslipidemia, in an endogamous population of North India. However, the mechanism behind the same is still elusive. With this backdrop, we attempted to understand the reason for impaired homocysteine metabolism not resulting in significant CVD-associated morbidity and mortality rates in the same population.

Thus, we undertook this study to investigate the role of Foxp3 mRNA in blood and related plasma pro- and anti-atherogenic cytokines (IL-17A and IL-10) among individuals with impaired homocysteine metabolism (IHM group, plasma homocysteinemia ≥15 umol/L) in comparison to those with normal homocysteine metabolism (NHM group, plasma homocysteinemia <15 umol/L) in a folate replete and vitamin B12 deficient endogamous population cohort from Haryana state, India.

**Methodology**

**Study Population Demographic, Anthropometry and Sample Collection**

Ethical clearance was obtained from the Departmental Research Committee, Department of Anthropology, University of Delhi, India. Forty (40) normal, healthy individuals, between aged 45 and 65 years, unrelated up to first cousins, belonging to the Jat community, were randomly recruited from two villages (Mitrol and Aurangabad) of Palwal district, Haryana, India. All study participants were subjected to demographic and physiological and anthropometric profiling. Intravenous blood samples (2 mL in EDTA and 2 mL in RNA stabilisation buffer (Roche)) were collected from all individuals with prior written informed consent. Plasma separation was done, followed by DNA and RNA isolation, and was stored at -80° until assayed.

**Biochemical Estimations and Polymorphism Detection**

Plasma homocysteine, folate and vitamin B12 estimations were done using Chemiluminescence (Immulite Immunoassay system: Immulite® 1000; Catalog Nos. LKHO1, L2KFO2 & LKVBO1, respectively). Impaired homocysteine metabolism (IHM group) was defined as plasma homocysteine levels ≥15µmol, and study participants with normal homocysteine metabolism (plasma homocysteine <15µmol/L) were grouped as NHM group. Plasma IL-10 and IL-17A levels were measured using Quantikine ELISA Human IL-10 and IL-17 ImmunoAssay (R&D systems; Catalog Nos. D1000B & D1700 respectively). DNA isolation was done using salt- ing out precipitation method. MTHFR C677T polymorphism (rs1801133) was detected using a previously described protocol.

**Foxp3 Quantitative Reverse Transcriptase Polymerase Chain Reaction**

The stabilized blood samples (in “RNA/DNA stabilization reagent for blood/bone marrow”; Roche, catalog No. 11934317001) were subjected to total RNA isolation (High Pure RNA isolation kit, Roche: 11 823 65 001) followed by cDNA conversion using random hexamer primers as per manufacturer’s protocol (Transcriptor first strand cDNA synthesis Kit, Roche: 04 379 012 001). The cDNA was then assessed...
using real-time quantitative polymerase chain reaction (Roche) using Sybr green technology. Each sample was assessed in triplicates, both for Foxp3 (target gene) and for 18S (housekeeping gene). Primer sequences were taken from already published study. A number of negative controls were put in each assay. Samples with coefficient of variation <5% for the technical replicates of both Foxp3 and 18S gene were used for data analysis. Prior to this, PCR efficiencies were also checked for both Foxp3 and 18S, and it was found that in blood, the expression of Foxp3 was low. The relative expression of Foxp3, normalized to 18S, was calculated and expressed as $2^{\Delta CT}$ method, as per Schmittgen & Livak.

Statistical Analysis

For all continuous variables, values are reported as mean ± standard deviation (age, body mass index [BMI], lipids, Foxp3 mRNA levels, homocysteine, folate, vitamin B12, interleukin 10, interleukin 17). For all categorical variables, numbers and percentages are reported. Independent t test and chi square test were used for comparison of continuous variables and categorical variables respectively in two groups. P <.05 was considered significant. All the variables were checked for normal distribution, and if found skewed then were log transformed (log to the base of 10). SPSS. Ver. 13 was used for the statistical analysis.

### Results and Discussion

The two groups (IHM and NHM) were comparable with respect to demographic, physiological and lipid variables except for LDL cholesterol, which was found to be relatively higher in IHM (though not significant) (Table 1). Vitamin B12 deficiency was equally high in both groups (~66%) (Table 1). However, the study participants in IHM group also had folate deficiency (18%), unlike NHM group. Relatively lower levels of Foxp3 mRNA were found in IHM group but plasma IL-10 levels were comparable for both the groups. Conversely, significantly high plasma IL-17A levels were found in IHM group (Figure 1).

The low mRNA levels of Foxp3 in the IHM group (though not significant) are in line with a previous study, which reported a that hyperhomocysteinemic ApoE deficient mouse model suppressed the function of Foxp3 mRNA and protein expression. This could be due to the combinational effect of hyperhomocysteinemia and significant ApoE deficiency in the murine model, which did not replicate in our study. Further, Kinoshita et al reported the promotion of Foxp3+ Treg cells through dietary folate and Yamaguchi et al showed Tregs express folate receptors, and do not survive in folate deficiency. Intriguingly, the present population, despite being folate replete showed lower levels of Foxp3, which could be attributed to vitamin B12 deficiency, consequently a folate trap. In addition, lower Foxp3 mRNA levels in the IHM group could

| Table 1. Distribution of variables related to demographics, physiological characteristics, lipids, and homocysteine metabolism in normal (NHM group) vs hyperhomocysteinemic (IHM group) individuals |
|---------------------------------|-----------------|-----------------|
| Parameters (Mean ± SEM)         | NHM Group       | IHM Group       |
| Demographic                     |                 |                 |
| Age, years                      | 45.64±3.02      | 45.80±1.57      |
| Sex (M/F)                       | 10(1/9)         | 30 (10/20)      |
| Smoking status (non-smoker/smoker) | 10 (7/3)     | 30 (18/12)      |
| Alcohol consumption (Yes/No)    | Nil             | 30 (28/2)       |
| Physiological characteristics   |                 |                 |
| Body mass index, BMI, kg/m²     | 24.67±1.47      | 24.32±0.75      |
| Systolic blood pressure, mm Hg  | 131.27±4.95     | 122.73±2.71     |
| Diastolic blood pressure, mm Hg | 84.45±1.25      | 79.43±2.38      |
| Lipids                          |                 |                 |
| Total cholesterol, mg/dL        | 187.53±11.49    | 201.64±9.85     |
| Triglycerides, mg/dL            | 147.75±20.08    | 105.63±9.9      |
| HDL, mg/dL                      | 44.88±2.89      | 46.71±2.44      |
| LDL, mg/dL                      | 113.1±10.14     | 133.81±8.5      |
| Non-HDLc, mg/dL                 | 142.66±12.22    | 154.93±9.42     |
| Homocysteine metabolism related markers |              |                 |
| MTHFR 677T allele frequency (CC/CT/TT) | 9 (8/1/0) (5%)  | 28 (18/9/1) (19.6%) |
| Plasma folate deficiency, <3ng/mL| Nil            | 18%            |
| Plasma vitamin B12 deficiency, <150pg/mL | 66%            | 66%            |
be due to homocysteine dependent DNA methylation changes on Foxp3, whose expression is controlled by DNA methylation at TSDR\(^2\) (TSDR-Treg Specific Demethylated Region, an evolutionary conserved region). Yang et al also demonstrated that hydrogen sulphide (also a by-product of impaired homocysteine-trans sulphuration pathway) promotes Foxp3 demethylation.\(^2\) Further, computational analysis of the MTHFR gene revealed putative TFBS (transcription factor binding sites) of Foxp3 in its differentially methylated region near TSS.\(^2\) Studies also indicate the influence of DNA methylation at IL-10 gene promoter influences phospho-CREB binding, consequently affecting the transcriptional regulation and protein levels.\(^2\) Thus, investigating the methylation levels at MTHFR DMR, Foxp3 TSDR and IL-10 promoter region would be interesting to understand the gene-gene and gene nutrition interaction of athero-immunological paradigm and homocysteine metabolism. Importantly, plasma IL-17A levels were significantly high in the IHM group and are in agreement with the previous report on the murine model.\(^1\) Further, hyperhomocysteinemia induced IL-17A levels have been linked through N-Sun2 pathway in rat T lymphocytes.\(^1\) IL-17A is considered as a pro-atherogenic, pro-inflammatory, signature cytokine of Th17 cells and is involved in the formation of foamy macrophages; blockade of IL-17A prevents the atherosclerotic

![Graphs showing Plasma homocysteine, interleukin 17A, interleukin 10, and Foxp3 mRNA levels in normal (NHM group) and hyperhomocysteinemic (IHM group) study participants. Significant differences denoted as a. <.001, b. <.0001.]
lesion progression. Also, Varshney et al demonstrated that high levels of IL-17A results in cholesterol accumulation in keratinocytes. In our present study, we also observed relatively high levels of LDL in the IHM group, suggesting a pre-morbid condition in impaired homocysteine metabolism. Further, high homocysteine has been shown to increase the production of ROS in T lymphocytes and induces the production of oxidised LDL.

Thus, the findings of our present study suggest that impaired homocysteine metabolism, in terms of hyperhomocysteinemia, could have resulted in relatively high LDL/dyslipidemia-like phenotype and significantly disturbed IL-17 signalling high IL-17A levels. However, the normal levels of plasma IL-10 levels, which could be attributed to folate repletion, indicate a balancing act between pro- and anti-atherogenic markers studied (IL-10 and IL-17A). This reveals one of the mechanisms for lower cardiovascular adversities, despite such a high frequency of hyperhomocysteinemia reported in the studied population through the balancing act or protective effect of an anti-atherogenic marker (IL-10) (as also reviewed by Mallat et al) toward pro-atherogenic state (high IL-17A). However additional studies are necessary to validate these findings. This also suggests the potential benefits of using this same lens in studying populations with concurrence of high rates of hyperhomocysteinemia.

CONCLUSION

In conclusion, our present findings suggest that the studied population (Jats of Haryana, North India) and similar populations exhibiting high levels of homocysteine but folate repletion and who might also carry high IL-17A pro-atherogenic marker, may have an increased burden of pre-morbid conditions. However, these conditions do not reach to significant mortality/morbidity, which is attributed to the counter-action or balancing act of IL-10, an anti-atherogenic marker. This further underscores the need to assess environment-influenced epigenetic control mechanisms of the targeted genes in the population studied through our research.

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