3.1 Introduction

Vitiligo is one of the most common cosmetic disfigurement disorders caused due to loss of functional melanocytes from the epidermis (Poole *et al.,* 1993). The precise *modus operandi* for vitiligo pathogenesis has remained obscure, however, certain genetic predisposition factors and several potential precipitating events such as neurochemical, oxidative stress, autoimmunity etc. were found to be involved (Laddha *et al.,* 2013). Various pro-oxidants generated during melanin synthesis and intrinsic antioxidant defense mechanisms that are compromised in pathologic conditions make epidermal melanocytes more vulnerable to oxidative stress (Denat *et al.,* 2014). In addition, the oxidative stress acts as the triggering event in progressive melanocyte destruction in vitiligo (Laddha *et al.,* 2014). Elevated homocysteine levels are associated with various diseases including cardiovascular, neurodegenerative autoimmune diseases and diabetes mellitus (Schalinske and Smazal 2012; Huang *et al.,* 2013). Several studies have reported increased homocysteine (Hcy), and reduced vitamin B_{12} and folic acid levels in vitiligo patients (Montes *et al.,* 1992; Kim *et al.,* 1999; Shaker and El-Tahlawi 2008; Silverberg and Silverberg 2011; Karadag *et al.,* 2012). It was also reported that treating vitiligo patients with vitamin B_{12} and folic acid showed signs of improvement in their condition (Juhlin and Olsson 1997). Both vitamin B_{12} and folic acid act as cofactors for the enzymes involved in the regeneration of methionine from homocysteine (Mudd *et al.,* 2001). Consequently, nutritional deficiency of these two vitamins results in an increase in homocysteine and a decrease in methionine levels (Guttormsen *et al.,* 1996; Miner *et al.,* 2000). Methylenetetrahydrofolate reductase (MTHFR) is an important regulatory enzyme involved in the conversion of homocysteine to methionine. Polymorphisms in *MTHFR* i.e., 677 C>T (rs1801133) and 1298 A>C (rs1801131) result in decreased activity of MTHFR enzyme and affect Hcy levels (Böttiger *et al.,* 2007; Hustad *et al.,* 2007; Misra *et al.,* 2010). We have observed significant association of *MTHFR* rs1801131 polymorphism with vitiligo susceptibility in Gujarat population (Jadeja *et al.,* 2018). From the above, we speculated a possible influence of *MTHFR* polymorphisms and vitamin B_{12} levels on homocysteine homeostasis in vitiligo patients. Hence, in the present study, we aimed to (i) estimate plasma homocysteine & vitamin B_{12} levels; (ii) investigate the correlation of *MTHFR* rs1801133 and rs1801131 polymorphisms with plasma Hcy levels; and (iii) estimate homocysteine levels from suction induced blister fluid samples from the skin of vitiligo patients and controls from Gujarat population.

3.2 Materials and methods

3.2.1 Selection of subjects for homocysteine and vitamin B_{12} estimation

For the estimation of homocysteine and vitamin B_{12} levels, 60 controls and 55 vitiligo patients were selected. Exclusion criteria for controls included were cigarette smoking, alcohol intake, pregnancy, vitamin intake, especially folic acid, B_6, B_{12} and hormonal therapy. Exclusion criteria for patients included were cigarette smoking, intake of folic acid, vitamin B_6, B_{12}, intake of medicines affecting homocysteine and vitamin B_{12} levels including topical or oral corticosteroids, phototherapy, hormonal therapy, etc., diseases known to affect the homocysteine levels including genetic disorders of amino acid metabolism, hypertension, diabetes mellitus, thyroid dysfunction, cardiovascular disease, renal failure, deep venous thrombosis, psoriasis and pregnancy. Patients and controls were of the same ethnicity. After taking complete history, general and dermatological examination of patients and controls was carried out. Vitiligo patients who fulfilled the inclusion criteria were enrolled in the study. The demographic details of the subjects recruited for the estimation of homocysteine and vitamin B_{12} levels are shown in Table 3.1.

Table 3.1. Demographic characteristics of vitiligo patients and controls recruited for homocysteine and vitamin B_{12} estimation.

	Vitiligo Patients	**Controls**
	(n = 55)	(n = 60)
Average age (mean age ± SD)	33.15 ± 14.13 yr	30.78 ± 11.15 yr
Sex: male	26 (47.27 %)	28 (46.67 %)
female	29 (52.72 %)	32 (53.33 %)
Onset age (mean age ± SD)	26.57 ± 15.52 yr	NA
Duration of disease (mean ± SD)	7.17 ± 5.96 yr	NA
Family history	12 (21.81 %)	NA
Type of disease		
Generalized (GV)	38	NA
Localized (LV)	17	NA
Active (AV)	39	NA
Stable (SV)	16	NA

3.2.2 Collection of blood samples

The importance of the study was explained to all the participants and written consent was obtained. After at least 12 hours of fasting, 5ml blood was drawn from each participant and collected in EDTA tubes. Blood samples were centrifuged (3000 rpm for 5 min at 4^oC) to separate the plasma. Two aliquots of each sample were made under the light protected conditions and stored at -80^o C until the time of analysis.

3.2.3 Collection of suction induced blister fluid samples

A detailed methodology of suction induced blister fluid sample collection from skin of vitiligo patients (n=15) and controls (n=18) is described in **section 2.2.10** of chapter 2.

3.2.4 Estimation of homocysteine and vitamin B_{12} levels

Homocysteine levels from plasma and suction induced blister fluid samples were estimated by ECLIA using Immulite^{TM} 2000 (DPC, United States). Plasma vitamin B_{12} levels were estimated by ECLIA using Cobas^{TM} e411 (Roche diagnostics, USA).

3.2.5 Genotyping of MTHFR rs1801133 and rs1801131 polymorphisms

MTHFR rs1801133 and rs1801131 polymorphisms were genotyped by PCR-RFLP and ARMS-PCR methods respectively. Other details are described in **section 2.2.5 and section 2.2.6** of chapter 2.

3.2.6. Statistical analyses

Analysis of homocysteine and vitamin B_{12} levels in patients and controls was carried out using unpaired t-test and one-way ANOVA. Tukey's multiple comparison test was applied for multiple testing. *p*-value less than 0.05 was considered to be significant. All the statistical tests were carried out using Prism 6 software (Graph Pad Software, USA).

3.3. Results:

3.3.1. Analysis of homocysteine levels

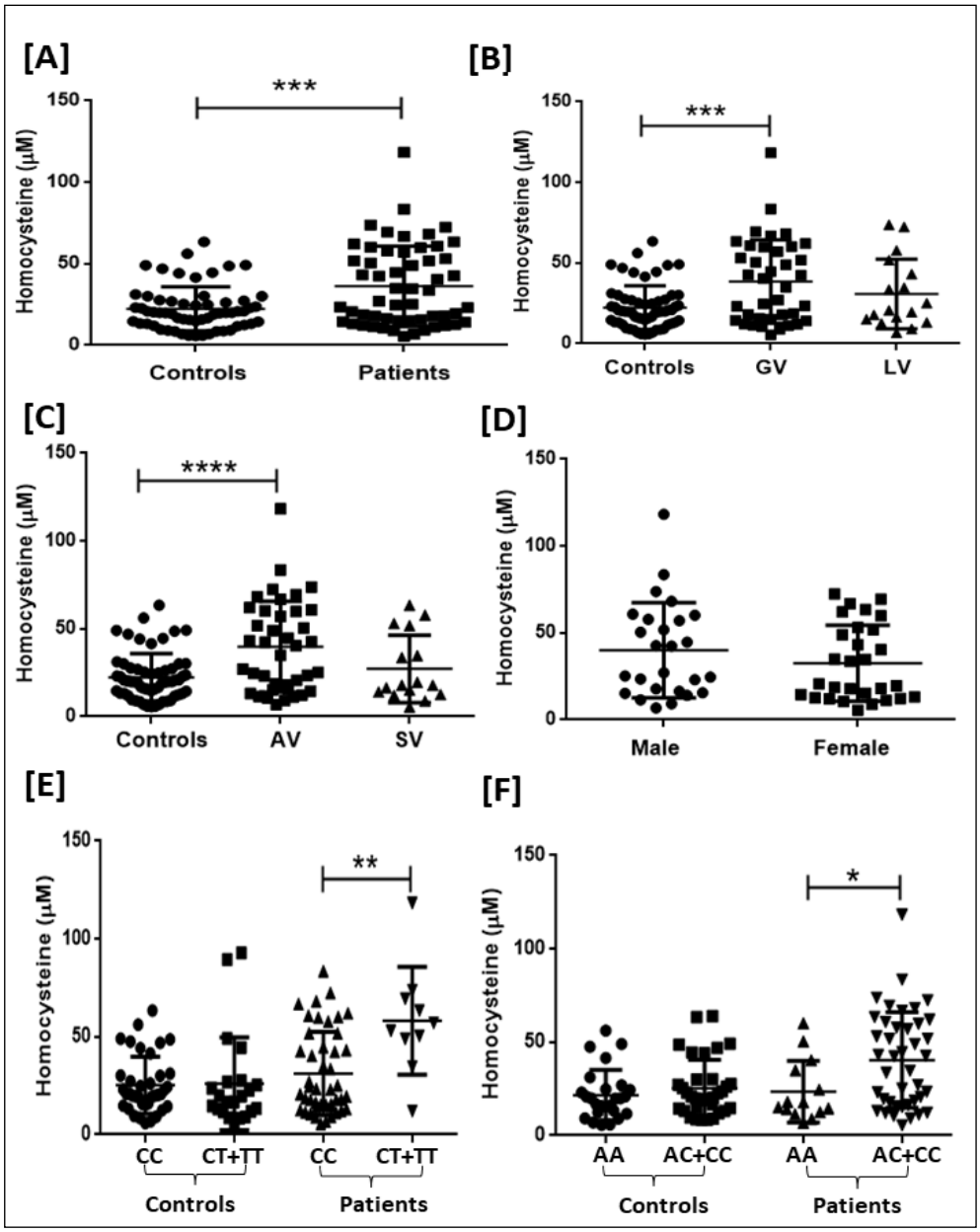

Figure 3.1: Estimation of plasma homocysteine levels in vitiligo patients (n=55) and controls (n=60). [A] Vitiligo patients showed significantly elevated homocysteine levels as compared to controls (p=0.0003). [B & C] Analysis based on type and activity of vitiligo revealed significantly elevated homocysteine levels in GV as well as AV patients as compared to controls (p=0.0004 and p=<0.0001, respectively. [D] Analysis based on gender revealed no significant difference in homocysteine levels between male and female patients with vitiligo (p=0.2671). [E & F] Significantly elevated homocysteine levels were observed in patients with vitiligo carrying *MTHFR* rs1801133 CT+TT genotypes and *MTHFR* rs1801131 AC+CC genotypes as compared to the respective ancestral genotypes (p=0.0012 and p=0.0255 respectively). However, no significant difference in homocysteine levels was observed in controls with respect to *MTHFR* rs1801133 and rs1801131 polymorphisms.

Plasma homocysteine levels were assessed in 60 controls and 55 vitiligo patients by unpaired t-test (Figure 3.1A). Vitiligo patients showed significant increase in plasma homocysteine levels as compared to controls (mean ± SEM: 36.13 ± 3.322 µM vs 22.44 ± 1.735 µM, respectively; *p*=0.003). Analysis of homocysteine levels based on type of vitiligo in 60 controls, 38 GV patients, and 17 LV patients was carried out by one-way ANOVA (Figure 3.1B). A significant increase in homocysteine levels was observed in GV patients as compared to controls (mean ± SEM: 38.50 ± 4.182 µM vs 22.44 ± 1.735 µM, respectively; *p*=0.0004). However, there was no significant difference in homocysteine levels between GV and LV patients (mean ± SEM: 38.50 ± 4.182 µM vs 30.83 ± 5.249 µM, respectively; *p*=0.372) as well as in LV patients as compared to controls (mean ± SEM: 30.83 ± 5.249 µM vs 22.44 ± 1.735 µM, respectively; *p*=0.0266). Further, analysis of homocysteine levels was carried out based on type of vitiligo in 60 controls, 39 AV patients, and 16 SV patients by one-way ANOVA (Figure 3.1C). Significant increase in homocysteine levels was observed in AV patients as compared to controls (mean ± SEM: 39.82 ± 4.141 vs 22.44 ± 1.735 µM, respectively; *p*=<0.0001). However, there was no significant difference in homocysteine levels between AV and SV patients (mean ± SEM: 39.82 ± 4.141 vs 27.15 ± 4.806 µM, respectively; *p*=0.0731) as well as in SV patients as compared to controls (mean ± SEM: 27.15 ± 4.806 vs 22.44 ± 1.735 µM, respectively; *p*=0.6613). Homocysteine levels were analysed with respect to sex differences in 26 male and 29 female patients by unpaired t-test (Figure 3.1D). No significant difference was observed in both the groups (mean ± SEM: 40.06 ± 5.361 µM vs 32.61 ± 4.052 µM, respectively; *p*=0.2671).

3.3.2 Genotype-phenotype correlation for *MTHFR* rs1801133 and rs1801131 polymorphisms

MTHFR rs1801133 and rs1801131 polymorphisms were reported to show decreased MTHFR activity and influence homocysteine levels (Böttiger *et al.,* 2007; Hustad *et al.,* 2007; Misra *et al.,* 2010). Hence, we have analysed the homocysteine levels in individuals with respect to their genotype. Homocysteine levels were analysed with respect to *MTHFR* rs1801133 polymorphism in controls and patients by unpaired t-test (Figure 3.1E). Homocysteine levels were significantly higher in patients carrying CT and TT genotypes as compared to those carrying CC genotypes (mean ± SEM: 58.13 ± 8.733 µM vs 31.24 ± 3.178 µM, respectively; *p*=0.0012). However, no significant difference was observed between controls carrying CC genotype and those carrying CT and TT genotypes (mean ± SEM: 25.29 ± 2.350 µM vs 26.01

± 5.057 µM, respectively; *p*=0.8837). Homocysteine levels were also analysed with respect to *MTHFR* rs1801131 polymorphism in controls and patients by unpaired t-test (Figure 3.1F). Homocysteine levels were significantly higher in patients carrying AC and CC genotypes as compared to those carrying AA genotype (mean ± SEM: 40.43 ± 4.001 µM vs 23.55 ± 4.413, respectively; *p*=0.0255). However, no significant difference was observed between controls carrying AA genotype and those carrying AC and CC genotypes (mean ± SEM: 21.71 ± 2.652 µM vs 25.28 ± 2.629 µM, respectively; *p*=0.3515).

3.3.3 Estimation of Vitamin B_{12} levels

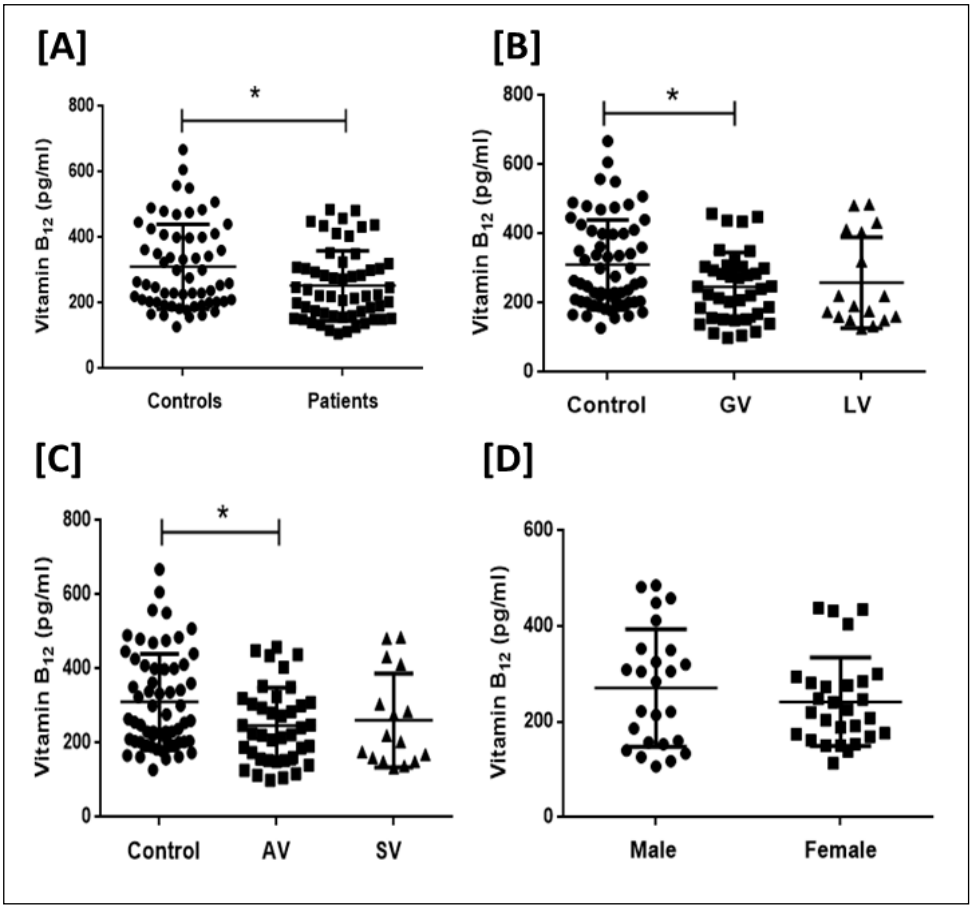

Figure 3.2: Estimation of plasma vitamin B_{12} levels in vitiligo patients (n=55) and controls (n=60). [A] A significant decrease in vitamin B_{12} levels was observed in patients as compared to controls (*p*=0.0102). [B] Analysis of different types of vitiligo suggested significantly reduced vitamin B_{12} levels in GV patients as compared to controls (*p*=0.033). No significant difference was observed in GV and LV as well as in LV patients as compared to controls (*p*=0.945 and *p*=0.260, respectively). [C] Analysis of vitamin B_{12} levels based on the activity of vitiligo suggested a significant decrease in AV patients as compared to controls (*p*=0.029). There was no significant difference in AV and SV patients as well as in SV patients as compared to controls (*p*=0.311 and *p*=0.914, respectively). [D] No significant difference in vitamin B_{12} levels was observed between male and female vitiligo patients (*p*=0.3313).

Vitamin B_{12} levels in plasma samples of 60 controls and 55 vitiligo patients were analysed by unpaired t-test (Figure 3.2A). Significantly lower vitamin B_{12} levels were observed in vitiligo patients as compared to controls (mean ± SEM: 252.8 ± 14.43 pg/ml vs 311.0 ± 16.74 pg/ml, respectively; p=0.0102). Analysis of vitamin B_{12} levels based on type of vitiligo in 60 controls, 38 GV patients, and 17 LV patients was done by using one-way ANOVA (Figure 3.2B). Significant decrease in vitamin B_{12} levels was observed in GV patients as compared to controls (mean ± SEM: 247.4 ± 16.0 pg/ml vs 311.0 ± 16.74 pg/ml, respectively; p=0.0331). However, there was no significant difference in vitamin B_{12} levels between GV and LV patients (mean ± SEM: 247.4 ± 16.0 pg/ml vs 258.7 ± 31.94 pg/ml, respectively; p=0.9449) as well as in LV patients as compared to controls (mean ± SEM: 258.7 ± 31.94 pg/ml vs 311.0 ± 16.74 pg/ml, respectively; p=0.2602). Analysis of vitamin B_{12} levels based on type of vitiligo in 60 controls, 39 AV patients, and 16 SV patients was carried out by one-way ANOVA (Figure 3.2C). Significant decrease in vitamin B_{12} levels was observed in AV patients as compared to controls (mean ± SEM: 246.7 ± 16.33 pg/ml vs 311.0 ± 16.74 pg/ml, respectively; p=0.0291). However, there was no significant difference in vitamin B_{12} levels between AV and SV patients (mean ± SEM: 246.7 ± 16.33 pg/ml vs 261.2 ± 31.68, respectively; p=0.9142) as well as in SV patients as compared to controls (mean ± SEM: 261.2 ± 31.68 vs 311.0 ± 16.74 pg/ml, respectively; p=0.3106). Vitamin B_{12} levels were analysed with respect to sex differences in 26 male and 29 female patients by unpaired t-test (Figure 3.2D). No significant difference was observed in vitamin B_{12} levels in male as compared to female vitiligo patients (mean ± SEM: 271.1 ± 24.55 pg/ml vs 242.0 ± 17.50 pg/ml, respectively; p=0.3313).

3.3.4 Analysis of homocysteine levels from suction induced blister fluid samples

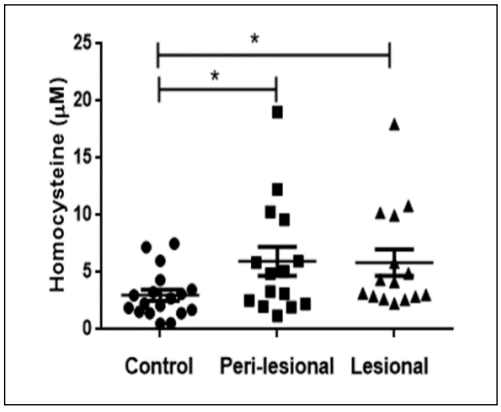

Figure 3.3: Estimation of homocysteine levels in suction induced blister fluid (SBF) samples of patients with active generalized vitiligo (n=15) and controls (n=18). Significant increase

was observed in homocysteine levels in SBF samples from perilesional skin (p=0.0363) and lesional skin (p=0.0430) of patients as compared to controls.

Homocysteine levels in suction induced blister fluid (SBF) samples collected from peri-lesional and lesional skin of 15 patients with active generalized vitiligo and from normal skin of 18 controls were analysed (Figure 3.3). The results suggest a significant increase in SBF homocysteine levels of peri-lesional (mean ± SEM: 5.96 ± 1.272 µM vs 3.00 ± 0.488 µM, respectively; p=0.0363) as well as lesional skin (mean ± SEM: 5.86 ± 1.154 µM vs 3.00 ± 0.488µM, respectively; p=0.0430) of active generalized vitiligo patients as compared to controls. However, no significant difference was observed in SBF homocysteine levels of peri-lesional skin as compared to lesional skin (mean ± SEM: 5.96 ± 1.272 µM vs 5.86 ± 1.154 µM, respectively; p=0.9431).

3.4 Discussion

Homocysteine has gained significant attention from researchers working on vitiligo for the last one decade. The present study reports significantly elevated plasma homocysteine levels as well as decreased vitamin B_{12} levels in vitiligo patients. Interestingly, the study also suggest significant increase in homocysteine levels in suction induced blister fluid samples from peri-lesional and lesional skin of vitiligo patients. Several studies also showed a significant elevation of homocysteine levels in active vitiligo patients as compared to stable vitiligo patients and also a positive correlation with the extent of depigmentation (Shaker and El-Tahlawi 2008; Singh *et al.*, 2011; Sabry *et al.*, 2014). In addition, suction induced blister fluid of active vitiligo patients was also reported to have elevated homocysteine levels (Anbar *et al.,* 2016). Homocysteine levels can be elevated by various constitutive, genetic and lifestyle factors, by inadequate nutrient status (vitamin B_6, B_9 & B_{12}) and due to various systemic diseases and medications (Strain *et al.,* 2004). Various studies have suggested decreased vitamin B_{12} levels in vitiligo, which may be a cause for hyper-homocysteinemia in vitiligo patients (Lecler D, Sibani S 2004; Park and Lee 2005). Many studies on vitamin B_{12} levels in vitiligo also have conflicting findings (Montes *et al.,* 1992; Kim *et al.,* 1999; Shaker and El-Tahlawi 2008; Yasar *et al.,* 2012; Al-Ghamdi *et al.,* 2014; Nahidi *et al.,* 2014a; Sabry *et al.,* 2014). Among genetic factors, *MTHFR* rs1801133 and rs1801131 polymorphisms are widely associated with elevated homocysteine levels (Brustolin *et al.,* 2010). Our recent study revealed a significant association of *MTHFR* rs1801131 polymorphism with generalized and

active vitiligo in Gujarat population (Jadeja *et al.,* 2018). *MTHFR* rs1801133 and rs1801131 polymorphisms lead to a significant reduction in MTHFR enzyme activity and influence the level of homocysteine (Lecler and Sibani, 2004). Interestingly, we observed that elevated homocysteine levels were significantly correlated to *MTHFR* polymorphisms in vitiligo patients. Hence, we speculate that *MTHFR* genotype and altered homocysteine & vitamin B_{12} levels may play a vital role in vitiligo pathogenesis. Homocystinuria has been reported to be associated with fair skin and hair, a phenomenon often described as 'pigmentary dilution' (Brenton *et al.,* 1966). Furthermore, it was suggested that homocysteine has an inhibitory effect on tyrosinase activity in the skin probably by binding with copper at the active site of the enzyme (Kurbanov *et al.,* 1974; Reish *et al.,* 1995). It has been also suggested that homocysteine metabolism may be altered by mutations in the catalase gene (Góth *et al.,* 2004). Besides, genetic polymorphisms of catalase and decreased catalase activity are associated with vitiligo (Casp *et al.,* 2002; Em *et al.,* 2007; Mansuri *et al.,* 2017). The production of toxic reactive oxygen species by homocysteine oxidation (Guilland *et al.,* 2003), together with other biochemical abnormalities in vitiligo such as biopterin metabolism, dysregulated antioxidant status, etc., might make the melanocytes vulnerable to oxidative stress that affect the melanogenesis (Schallreuter *et al.,* 1999; Agrawal *et al.,* 2004; Mansuri *et al.,* 2016a, 2017). Interestingly, elevated homocysteine levels, decreased vitamin B_{12} levels and *MTHFR* rs1801131 polymorphism are positively correlated with generalized and active vitiligo. Based on these findings, homocysteine seems to play a role in the autoimmune basis of vitiligo pathogenesis. Moreover, reports also suggest that homocysteine can induce production of various pro-inflammatory molecules such as MCP-1, IL-8, IL-6, IL-1β, etc. and also adhesion molecules, such as ICAM-1 (Dalal *et al.,* 2003; Su *et al.,* 2005; Lazzerini *et al.,* 2007; Nahidi *et al.,* 2014b). Previously, we have reported altered cytokine levels in vitiligo and the present study led us to speculate that homocysteine might be one of the contributing factors for the cytokine imbalance (Laddha *et al.,* 2012, 2013b; Dwivedi *et al.,* 2013; Singh *et al.,* 2018). Other harmful effects of homocysteine may be due to the reaction of homocysteine with proteins forming disulfides and the formation of highly reactive thiolactone (Ramakrishnan *et al.,* 2006). The accumulation of homocysteine-thiolactone is detrimental because of its intrinsic ability to modify proteins by forming N-homocysteine-protein adducts, in which a carboxyl group of homocysteine is N-linked to the ε-amino group of a protein lysine residue and it affects profoundly the protein structure and function (Jakubowski 2004). Due to N-homocysteinylation, proteins differ structurally when compared to native

proteins, hence they are likely recognized as neo self-antigens, thereby inducing an autoimmune response (Jakubowski 2004, 2005).

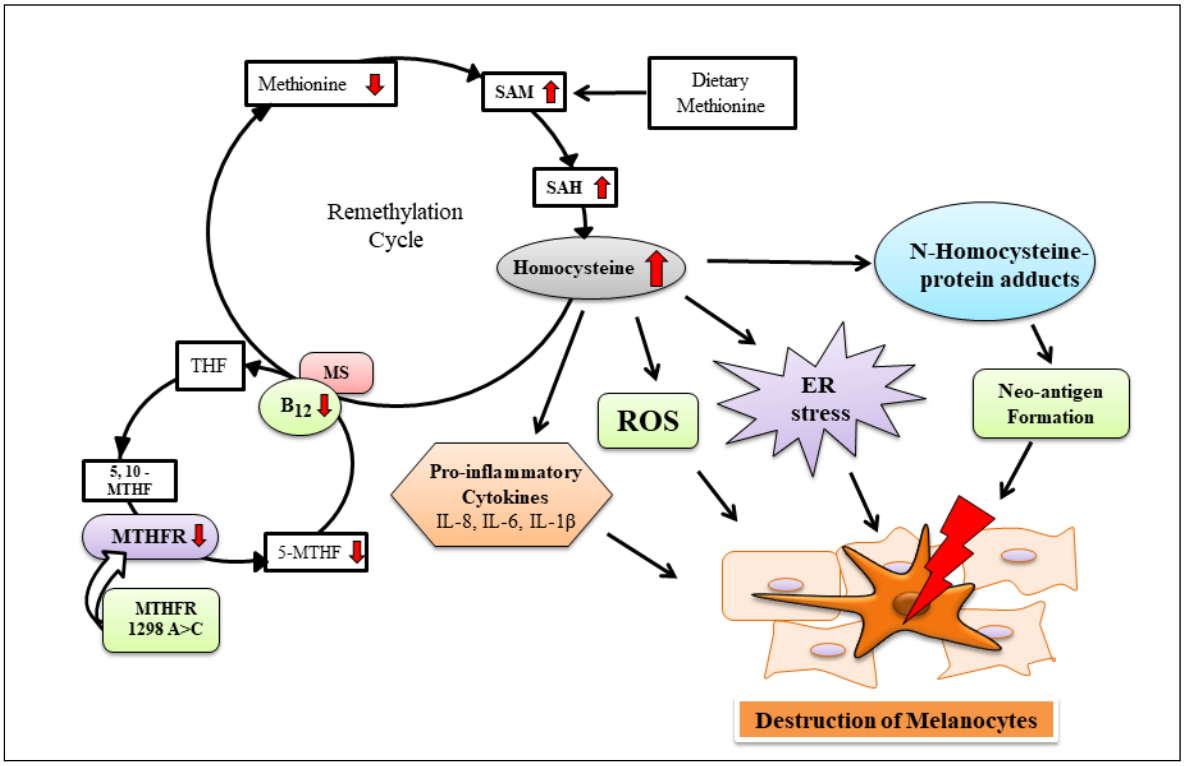

Figure 3.4: A possible mechanism for the role of homocysteine in melanocyte destruction in vitiligo. *MTHFR* 1298 A>C (rs1801131) polymorphism mediated reduction in MTHFR activity will affect the conversion of 5, 10-MTHF to 5-MTHF. A decrease in 5-MTHF along with reduced vitamin B_{12} levels results in reduction in remethylation of homocysteine to methionine. Decreased remethylation and continuous dietary methionine uptake will result in elevated homocysteine levels. Elevated homocysteine levels in the skin microenvironment might be an additional factor making melanocytes vulnerable. (MTHF: methyltetrahydrofolate, SAH: S-adenosyl-homocysteine; SAM: S-adenosyl-methionine THF: tetrahydrofolate)

Interestingly, a transcriptomic study of vitiliginous skin reported up-regulation of S-adenosylhomocysteine hydrolase (AHCY) gene which hydrolyzes S-adenosylhomocysteine (SAH) to adenosine and homocysteine (Dey-Rao and Sinha 2016). This further supports our findings. The skin microenvironment of vitiligo patients is already compromised due to several factors such as H_2O_2, increased proinflammatory cytokines, altered miRNA expression, etc. which makes the melanocytes vulnerable (Schallreuter *et al.*, 1999; Denat *et al.*, 2014; Mansuri *et al.*, 2016b). Earlier we have speculated that oxidative stress may be the initial triggering event to precipitate vitiligo. This is exacerbated by contributing autoimmune factors together with oxidative stress, and ER stress could be a potential link between

oxidative stress and autoimmunity in vitiligo (Laddha *et al.,* 2013; Mansuri *et al.,* 2014). Homocysteine is also known to induce endoplasmic reticulum (ER) stress response (Zhang *et al.,* 2001). The ability of homocysteine to induce oxidative stress, ER stress, inflammatory and immunomodulatory mechanisms suggest that homocysteine might be playing a vital role in initial triggering as well as the progression of vitiligo (Figure 3.4).

In conclusion, the present study suggests a possible role of altered homocysteine and vitamin B_{12} levels in precipitation and progression of vitiligo in genetically susceptible individuals. Besides, *MTHFR* 1 rs1801131 polymorphism is also found to be associated with autoimmune vitiligo. Correlating *MTHFR* polymorphisms with its enzyme activity in patients and controls would be interesting and further studies in this direction will throw light on the role of homocysteine in melanocyte biology and vitiligo pathogenesis.

3.5 References

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