3.1 INTRODUCTION

Vitiligo is an acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches caused due to loss of functional melanocytes from the epidermis. Generalized Vitiligo (GV) is considered to be an autoimmune disorder due its frequent association with other autoimmune diseases and presence of anti-melanocyte antibodies/autoreactive T cells in the circulation of vitiligo patients (Laddha *et al*., 2014). Cytokines have crucial functions in the development, differentiation and regulation of immune cells. As a result, dysregulation of cytokine production or their action is thought to have a central role in the development of autoimmunity (O'Shea, 2002). A large body of evidence suggests that epidermal melanocytes are an integral part of the skin immune system and can be considered as immunocompetent cells (Tam & Stępień, 2007). In addition to the photoprotective function, melanocytes also exhibit neuroendocrine activity and produce classical stress neurotransmitters, neuropeptides, cytokines and hormones (Slominski *et al*., 2009). Moreover in the epidermal melanin unit the surrounding keratinocytes synthesize cytokines such as IL-1α and IL-6, which are paracrine inhibitors of melanocyte proliferation and melanogenesis. This association with surrounding cells is of prime importance for melanocyte survival and differentiation as keratinocyte-derived cytokines act on melanocytes *via.* specific receptors (Moretti *et al.,* 2002). One of the emerging aspects of autoimmunity is epidermal cytokine imbalance which might pave the way for better understanding of vitiligo pathomechanisms.

Our results on selected candidate genes conferring oxidative stress and autoimmunity suggests that *HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLADRB1* 07:01* and studied polymorphisms in *IL4, CTLA4, SOD2, SOD3, GPX1, NALP1, MYG1, TNFA, TNFB, IFNG* and *IL10* genes are strongly associated with vitiligo susceptibility, whereas the polymorphisms in *PTPN22, MBL2, ACE, CAT, G6PD* and *SOD1* genes are not found to be significantly associated with vitiligo in Gujarat population (Begum *et al.,* 2014). Moreover, several reports including our studies have implicated cytokine imbalance in vitiligo pathogenesis (Mansuri *et al.,* 2014). Various reports as shown in Figure 1 have demonstrated association of cytokine and immune regulatory genes with vitiligo susceptibility (Laddha *et al*., 2014; Pehlivan *et al*., 2009; Lee *et al*., 1995; Imran *et al*.,

2012; Aydıngoz *et al*., 2015; Abanmi *et al*., 2008; Yun *et al*., 2010; Dwivedi *et al*., 2015; Namian *et al*., 2009, Alkhateeb *et al*., 2010).

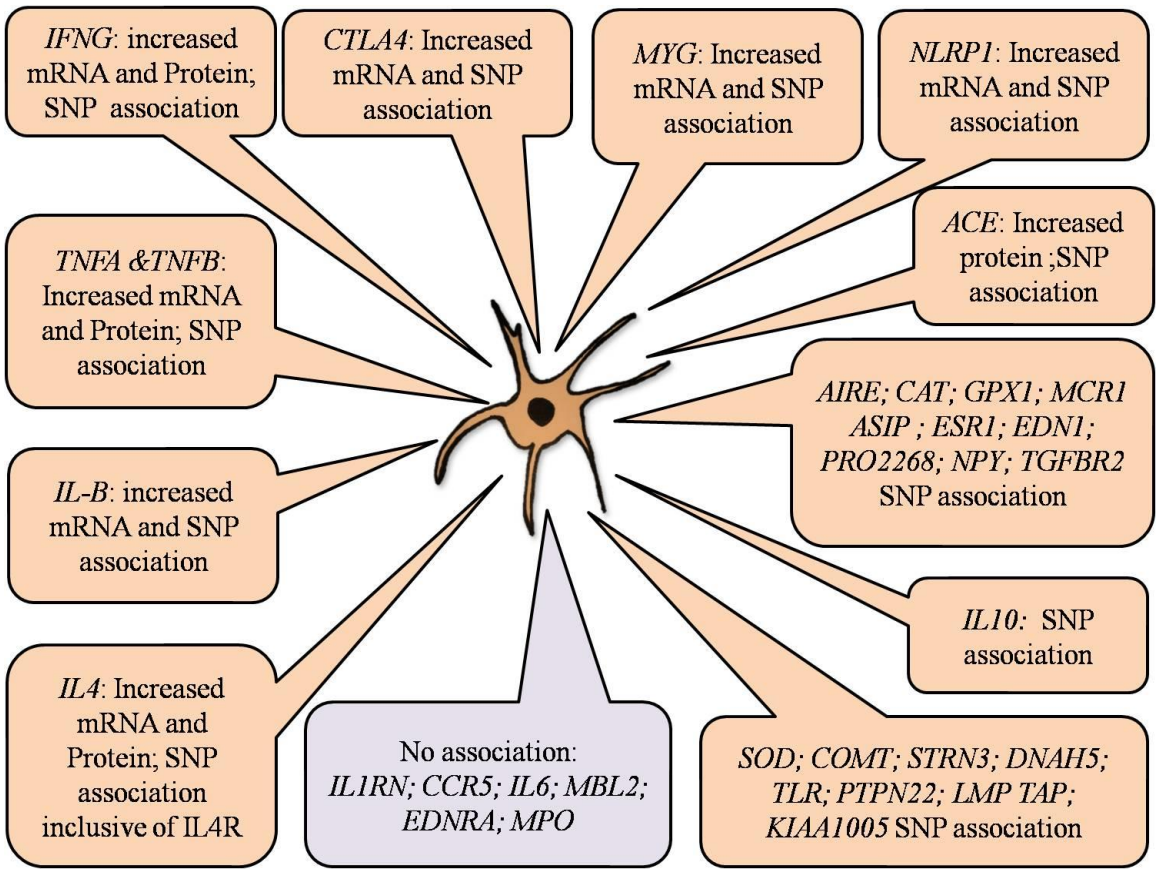

Figure 1: Illustration of SNPs association of candidate genes with vitiligo.

However, at present there is paucity of studies regarding cytokine expression in the skin samples derived from vitiligo patients. Therefore, we aim to monitor the expression profile of pro- and anti-inflammatory cytokines in lesional and non-lesional skin of vitiligo patients as compared to healthy controls. This study will provide better insights to understand the role of epidermal cytokine imbalance in the microenvironment of vitiliginous skin.

Various reports suggest that genetic predisposition of candidate genes which are involved in vitiligo susceptibility may affect the expression or function of respective genes (Figure 1). The target genes for the study were pro and anti-inflammatory cytokines: Tumor Necrosis Factor-α (*TNFA*), Interferon-γ (*IFNG*), Interleukin 1 Receptor 1 (*IL1R1*),

Interleukin-1α (*IL1A*), Interleukin-1β (*IL1B*), Interleukin-1 Receptor Antagonist (*IL1RN*), Interleukin-4 (*IL4*), Interleukin-6 (*IL6*) and Interleukin-10 (*IL10*). Results obtained from the proposed study might aid in understanding the role of epidermal cytokine imbalance in vitiligo pathogenesis.

3.2 MATERIALS AND METHODS

3.2.1 **Study Subjects**

Vitiligo patients who had not received systemic immunosuppressive treatment or PUVA/UVB, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy excision were recruited. Skin biopsies were taken from lesional and non-lesional skin of patients with vitiligo (n= 12) and from non-inflamed, non-irritated skin of healthy individuals (n= 12). Both vitiligo patients and healthy controls were of native Gujarat origin (Table 1). The importance of the study was explained to all participants and written consent was taken from controls and patients. The details regarding age, age of onset of vitiligo, duration of disease, family history, type of vitiligo, etc. of patients were filled in vitiligo questionnaire. The skin samples were procured by punch biopsy or from the left over skin after surgery. At the time of excision, the skin sample was collected in a sterile container with 20 ml of normal skin transporting medium containing Ca^{++} and Mg^{++} free Hank's balanced salt solution (HBSS) supplemented with 4X cocktail of penicillin, streptomycin and amphotericin B and kept at 4^0C while carrying it to cell culture laboratory. Immediately one part of the skin (from vitiligo patients and controls) was stored in RNA*later*® solution (Ambion, USA) for RNA isolation and gene expression studies. Samples obtained by methods other than punch biopsy were used for melanocyte culture.The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and conducted according to the Declaration of Helsinki's principles.

Table 1: Demographic characteristics of vitiligo patients and unaffected controls recruited for skin sample collection.

	Vitiligo Patients		**Controls**
	(n = 12)		(n = 12)
Average age (mean age ± SD)	29.38± 4.480		28.33± 3.240
Sex: Male	4		6
Female	8		6
Age of onset (mean age ± SD)	27.83 ± 6.05 yr		NA
Duration of disease (mean ± SD)	6.83 ± 2.36 yr		NA
Type of vitiligo			
Active vitiligo	7		NA
Stable vitiligo	5		NA

3.2.2 RNA isolation and 1st strand cDNA synthesis from skin samples

Skin samples stored in RNA *later*® solution (Ambion, USA) were used for RNA isolation. Skin samples were gradually thawed from -80^0 C to 4^0 C. The skin sample was homogenized in TriZol® solution under cooling conditions. RNA was isolated by using Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction method. RNA purity and integrity were assessed by the ratio of absorbance at 260 to 280 nm to be >1.95 and by 1.5% agarose gel electrophoresis/ethidium bromide staining, respectively. 1µg of RNA was reverse transcribed and converted to first strand cDNA using verso cDNA kit (Thermo scientific, USA) and cDNA was stored at -20^{0}C for gene expression studies.

Table 2: Details of primers used for expression of various candidate genes.

Primer	Sequence (5' to 3')	Amplicon size(bp)	Annealing Temperature (^{0}C)
TNFA FP	GCCCCCAGAGGGAAGAGTTCCCCA	124	70
TNFA RP	GCTTGAGGGTTTGCTACAACATGGGC		
IL1A FP	GCTGCTGAAGGAGATGCCTG	143	66
IL1A RP	CTACGCCTGGTTTTCCAGT		
IL1B FP	AGATGAAGTGCTCCTTCCAGG	153	65
IL1B RP	TGGTCGGAGATTCGTAGCTG		
IL4 FP	GCCTCCAAGAACACAACTGAGAAGG	212	68
IL4 RP	TCACAGGACAGGAATTCAAGCCCG		
IL6 FP	AAATTCGGTACATCCTCGACGGCA	88	61
IL6 RP	AGTGCCTCTTTGCTGCTTTCACAC		
IL10 FP	ACCTGGGTTGCCAAGCCTT	189	62
IL10 RP	CCACGGCCTTGCTCTTGTT		
IFNG FP	TTGGAAAGAGGAGAGTGACAG	212	65
IFNG RP	GGACATTCAAGTCAGTTACCGA		
IL1R1 FP	GGAGGCTGATAAATGCAAGG	195	62
IL1R1 RP	GTAAGATGAATTTACCACGC		
IL1RN FP	GCTGGAGGCAGTTAACATC	165	65
IL1RN RP	CTACTCGTCCTCCTGGAAG		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65
GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

3.2.3 Cytokine gene expression studies:

The expressions of candidate genes were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene

was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using Light Cycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification. The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out to check the specificity of the products formed. The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value. Cp values greater than 40 were excluded from the analysis. mRNA expression data was normalized with *GAPDH*.

3.2.4 Data analysis

ΔCp values were compared between different groups and data was analyzed by non-parametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine its statistical significance. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software (Faul *et al.,* 2007). *p*-values less than 0.05 were considered as statistically significant.

3.3 RESULTS

3.3.1 Gene expression studies of *TNFA* in control and vitiliginous human skin.

We performed a comparative analysis for *TNFA* expression in lesional and non-lesional skin from vitiligo patients and healthy skin from controls. Our results showed that lesional as well as non-lesional skin of vitiligo patients exhibit significant increase in expression of *TNFA* compared to control skin (p=0.024, p=0.0467 respectively). However, there was no significant difference in *TNFA* levels between lesional and non-lesional skin of vitiligo patients (p=0.6749; Figure 2).

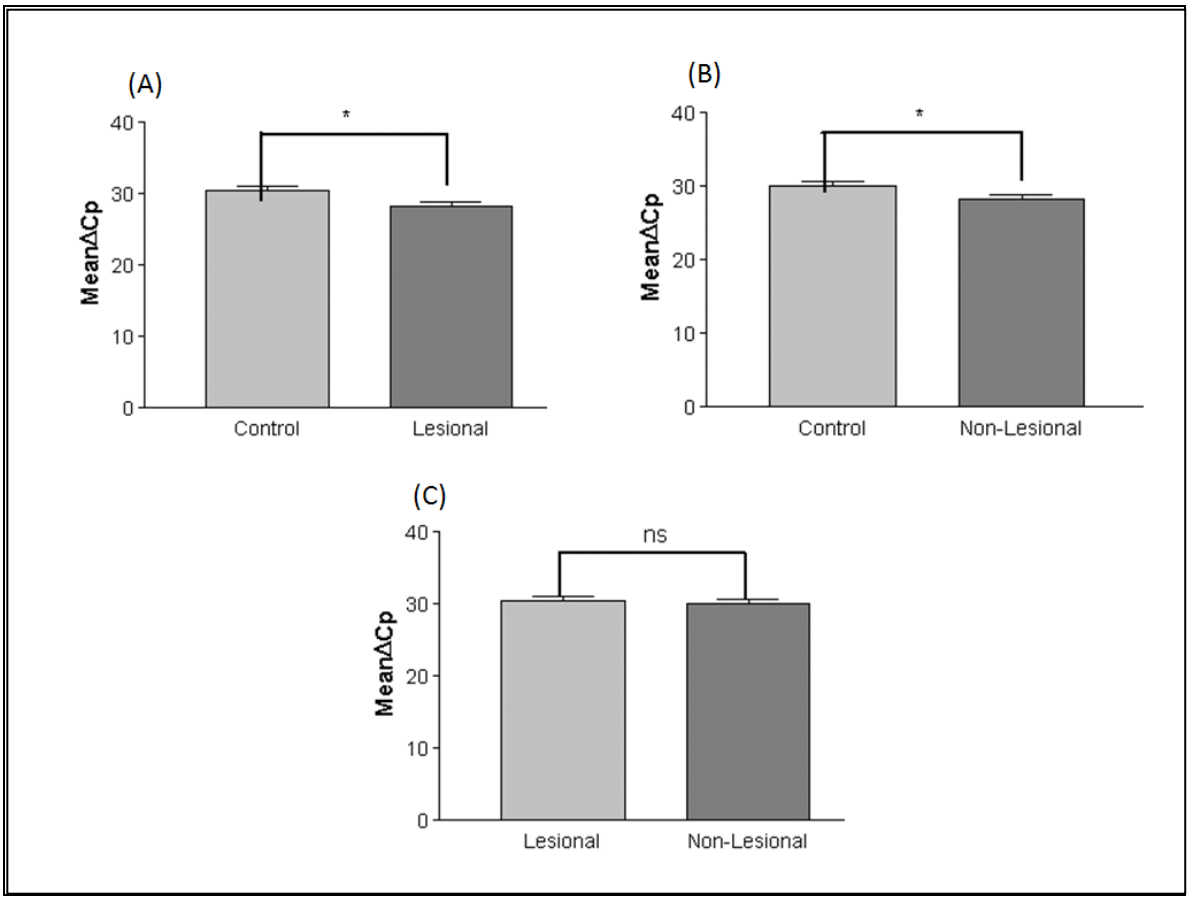

Figure 2: *TNFA* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant increase in expression of *TNFA* compared to control skin (p=0.0246).

(B) Non-lesional skin from vitiligo patient showed significant increase in expression of *TNFA* compared to control skin (p=0.0467).

(C) No significant difference was seen in *TNFA* levels between lesional and non-lesional skin of vitiligo patients (p=0.6749).

3.3.2 Gene expression studies of *IL1A* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1A* compared to control skin (p=0.6760

and p=0.4004, respectively). Also, there was no significant difference in *IL1A* levels between lesional and non-lesional skin of vitiligo patients (p=0.2886; Figure 3).

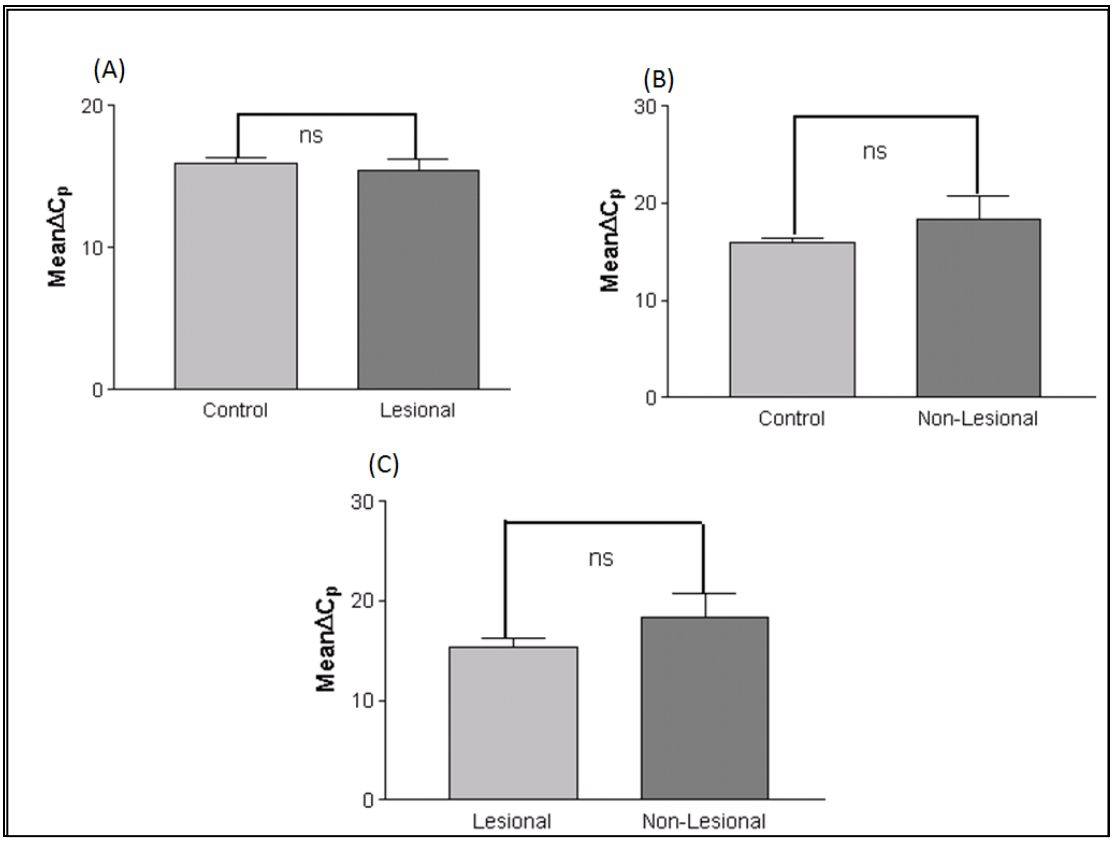

Figure 3: *IL1A* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1A* levels between lesional and control skin (p=0. 6000).

(B) No significant difference was seen in *IL1A* levels between non-lesional and control skin (p=0. 4004).

(C) No significant difference was seen in *IL1A* levels between lesional and non-lesional skin of vitiligo patients (p=0.2886).

3.3.3 Gene expression studies of *IL1B* in control and vitiliginous human skin.

Our results showed that lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1B* compared to control skin (p=0.5085). However, there

was a significant increase in *IL1B* levels in non-lesional compared to control skin (p=0.0290). Also significant increase in the expression of *IL1B* in non- lesional skin was observed compared to lesional skin of vitiligo patients (p=0.0021; Figure 4).

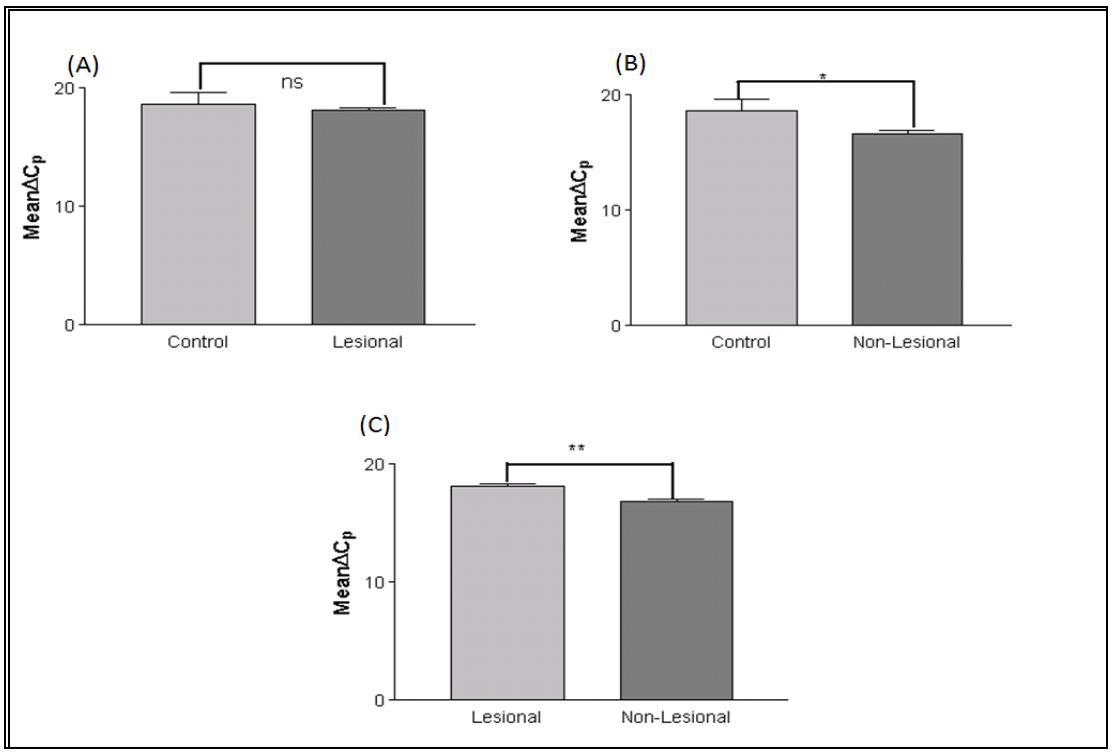

Figure 4: *IL1B* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1B* levels between lesional and control skin (p=0. 5085).

(B) Non-lesional skin from vitiligo patient showed significant increase in the expression of *IL1B* compared to control skin (p=0.0290).

(C) Significant increase was observed in *IL1B* levels in non-lesional compared to lesional skin of vitiligo patients (p=0.0021).

3.3.4 Gene expression studies of *IL4* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in the expression of *IL4* compared to control skin (p=0.2886

and p=0.2152, respectively). Further, there was no significant difference in *IL4* levels between lesional and non-lesional skin of vitiligo patients (p=0.9669; Figure 5).

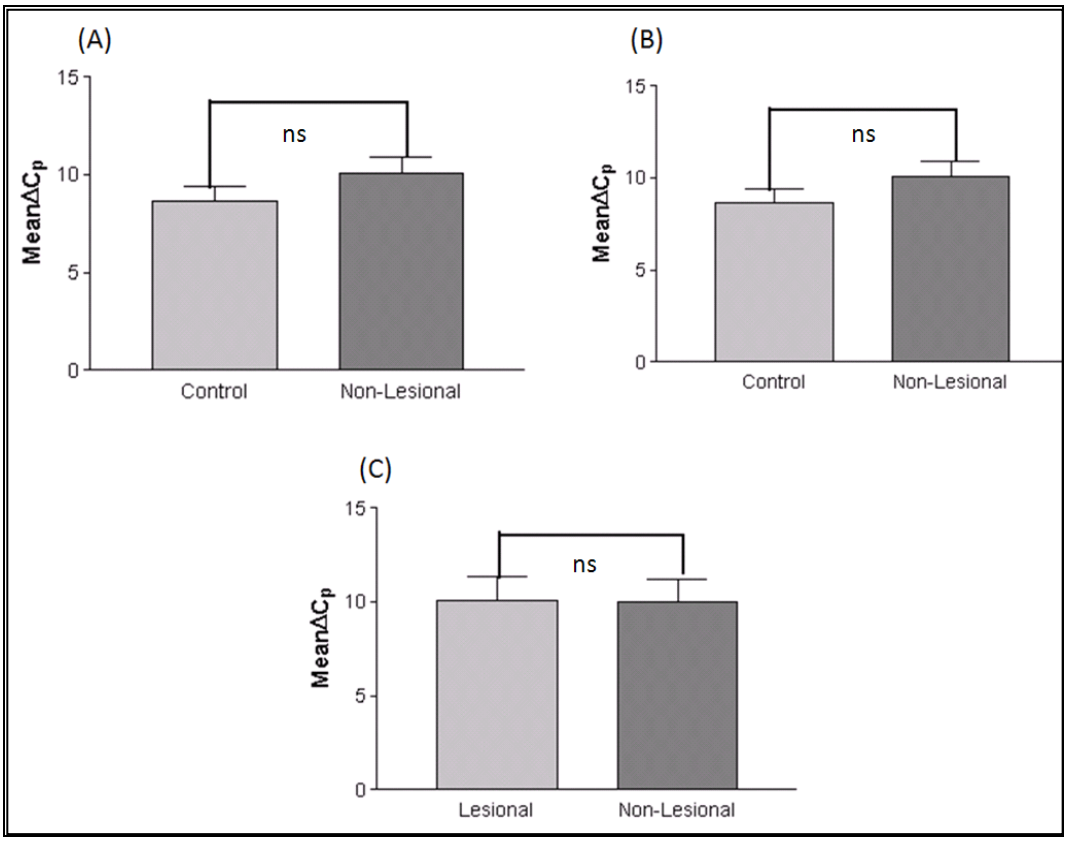

Figure 5: *IL4* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL4* levels between lesional and control skin (p=0. 2886).

(B) No significant difference was seen in *IL4* levels between non-lesional and control skin (p=0. 2152).

(C) No significant difference was observed in *IL4* levels between lesional and non-lesional skin of vitiligo patients (p=0.9669).

3.3.5 Gene expression studies of *IL6* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL6* compared to control skin (p=0.5473 and p=0.5697, respectively). Further, there was no significant difference in *IL6* levels between lesional and non-lesional skin of vitiligo patients (p=0.9511; Figure 6).

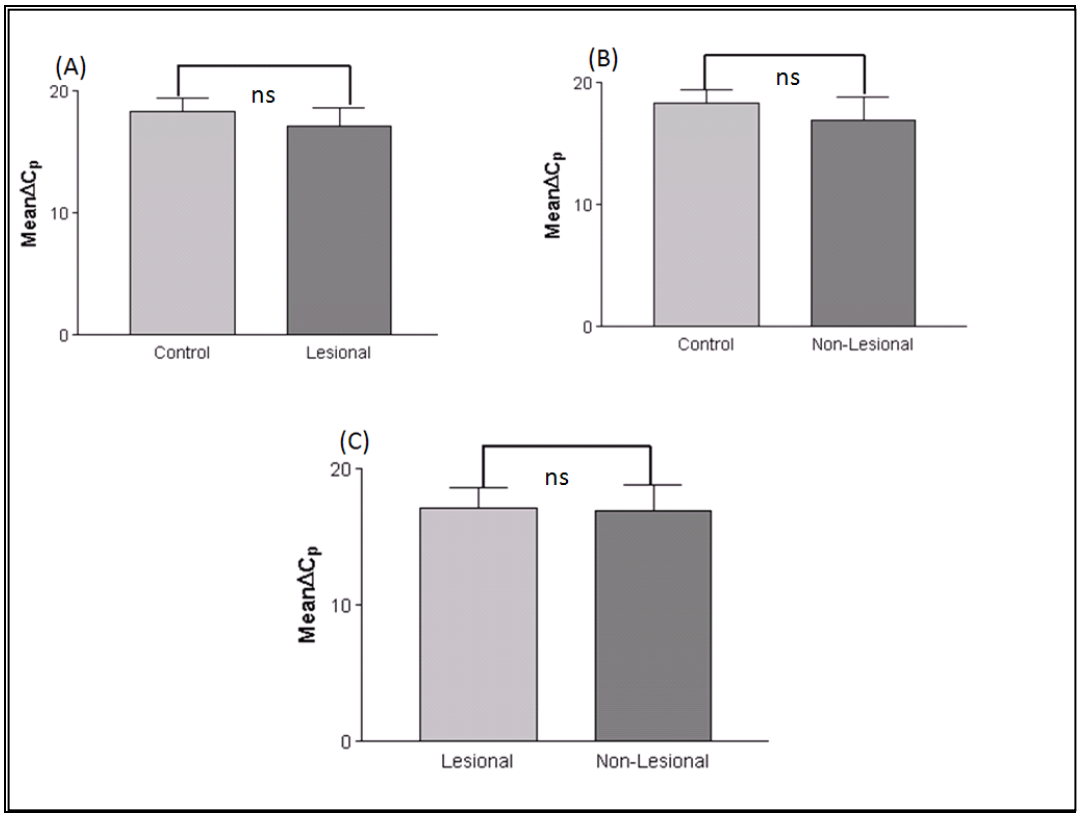

Figure 6: *IL6* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL6* levels between lesional and control skin (p=0.5473).

(B) No significant difference was seen in *IL6* levels between non-lesional and control skin (p=0. 5697).

(C) No significant difference was observed in *IL6* levels between lesional and non-lesional skin of vitiligo patients (p=0. 9511).

3.3.6 Gene expression studies of *IL10* in control and vitiliginous human skin.

IL10 levels were found to be reduced significantly in lesional skin of vitiligo patients compared to control skin (p=0.0357) while no significant difference was found in *IL10* levels between control and non-lesional skin (p=0.5565). Further, there was no significant difference in *IL10* levels between lesional and non-lesional skin of vitiligo patients (p=0.0980; Figure 7).

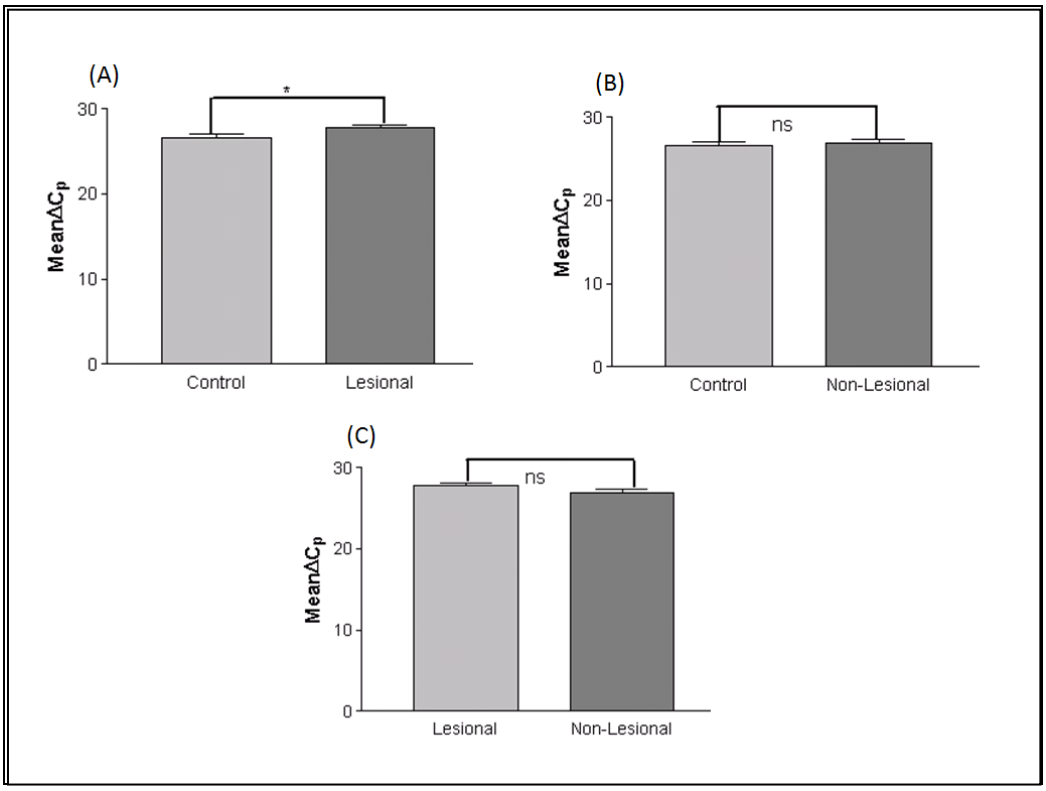

Figure 7: *IL10* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant decrease in the expression of *IL10* compared to control skin (p=0.0357).

(B) No significant difference was seen in *IL10 l*evels between non-lesional and control skin (p=0.5565).

(C) No significant difference was seen in *IL10* levels between lesional and non lesional skin of vitiligo patients (p=0.0980).

3.3.7 Gene expression studies of *IFNG* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients exhibited significant increase in expression of *IFNG* compared to control skin (p=0.0231 and p=0.0138, respectively). Further, there was no significant difference in *IFNG* levels between lesional and non-lesional skin of vitiligo patients (p=0.0808; Figure 8).

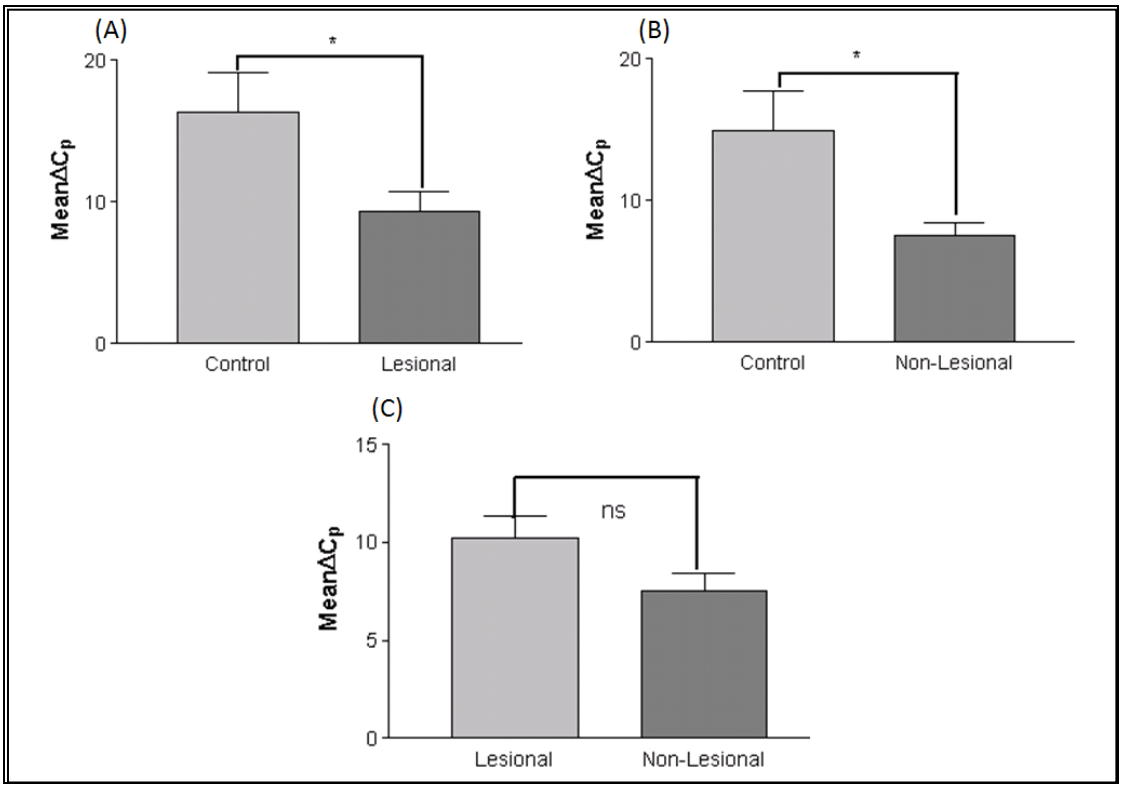

Figure 8: *IFNG* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant increase in the expression of *IFNG* compared to control skin (p=0.0231).

(B) Non-lesional skin from vitiligo patient showed significant increase in the expression of *IFNG* compared to control skin (p=0.0138).

(C) No significant difference was observed in *IFNG* levels between lesional and non-lesional skin of vitiligo patients (p=0.0808).

3.3.8 Gene expression studies of *IL1R1* in control and vitiliginous human skin.

We performed a comparative analysis for *IL1R1* expression between lesional and non-lesional skin from vitiligo patients and healthy skin from controls. Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1R1* compared to control skin (p=0.8186, p=0.2418 respectively). Further, there was no significant difference in *IL1R1* levels between lesional and non-lesional skin of vitiligo patients (p=0.8180; Figure 9).

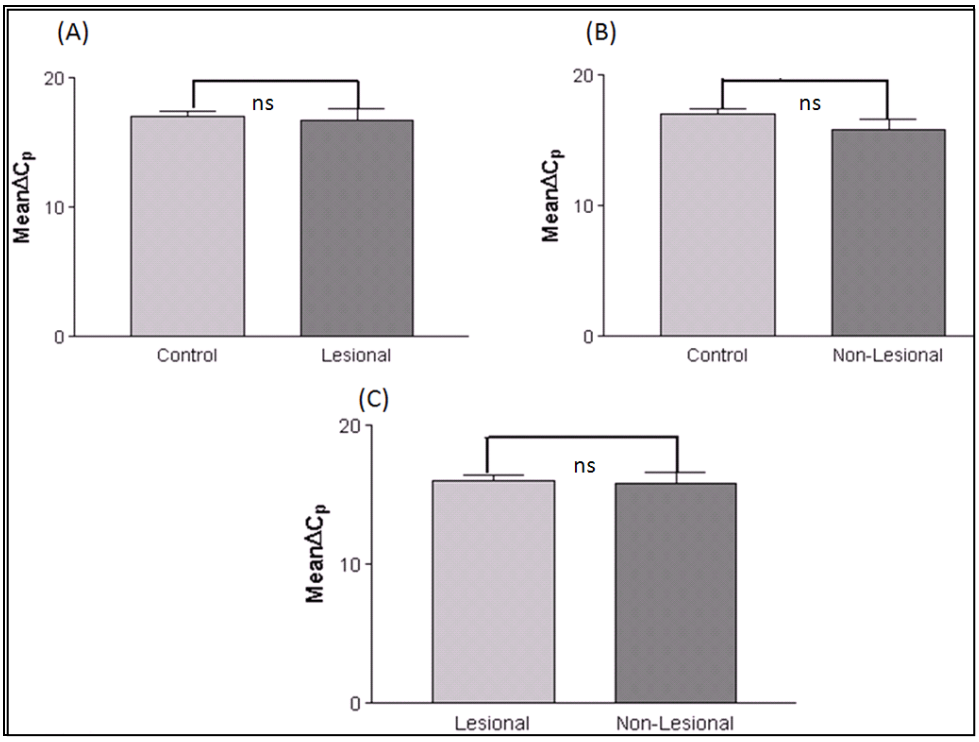

Figure 9: *IL1R1* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1R1* levels between lesional and control skin (p=0.8186).

(B) No significant difference was seen in *IL1R1 l*evels between non-lesional and control skin (p=0. 2418).

(C) No significant difference was seen in *IL1R1* levels between lesional and non-lesional skin of vitiligo patients (p=0.8180).

3.3.9 Gene expression studies of *IL1RN* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1RN* compared to control skin (p=0.2147 and p=0.8604, respectively). Further, there was no significant difference in *IL1RN* levels between lesional and non-lesional skin of vitiligo patients (p=0.4080; Figure 10).

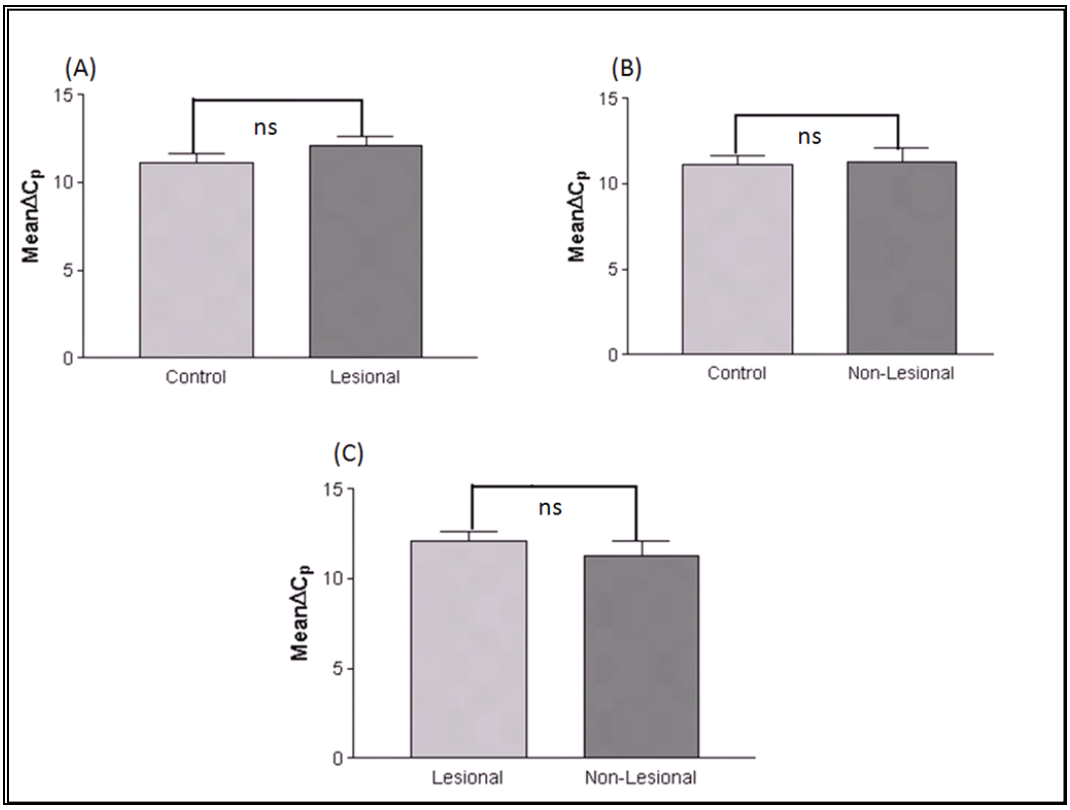

Figure 10: *IL1RN* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1RN* levels between lesional and control skin (p=0.2147).

(B) No significant difference was seen in *IL1RN* levels between non-lesional and control skin (p=0. 8604).

(C) No significant difference was observed in *IL1RN* levels between lesional and non-lesional skin of vitiligo patients (p=0. 4080).

Table 3: Analysis of transcripts levels of candidate genes in vitiligo patients and controls (n=12)

Gene	Lesional skin vs. Control (*p* value)	Non-lesional skin vs. Control (*p* value)	Non-lesional vs. lesional skin (*p* value)
TNFA	(0.0246) ↑	(0.0467) ↑	(0.6749) ns
IL1A	(0.6000) ns	(0.4004) ns	(0.2886) ns
IL1B	(0.5085) ns	(0.0290) ↑	(0.0021) ↑
IL4	(0.2886) ns	(0.2152) ns	(0.9669) ns
IL6	(0.5473) ns	(0.5697) ns	(0.9511) ns
IL10	(0.0357) ↓	(0.5565) ns	(0.0980) ns
IL1R1	(0.8186) ns	(0.2418) ns	(0.8180) ns
IL1RN	(0.2147) ns	(0.8604) ns	(0.4080) ns
IFNG	(0.0231) ↑	(0.0138) ↑	(0.0808) ns

3.4 DISCUSSION

Vitiligo is a multifactorial, polygenic skin disorder with involvement of various genes important for immune system, oxidative stress etc. (Laddha *et al.*, 2013). Immune responses in the skin involve an armamentarium of immune-competent cells and biological response modifiers including cytokines (Salmon *et al.*, 1994). Melanocytes are pigmentary cells residing at the basal layer of epidermis and contribute to cutaneous immune system. Melanocytes secrete various cytokines and express adhesion molecules upon exposure to different stimuli for e.g. cytokines, H_2O_2 etc. (Dwivedi *et al.*, 2013).

Moreover, in the epidermal melanin unit neighboring keratinocytes secrete several growth factors affecting melanocyte proliferation and melanin synthesis (Abdel-Naser *et al*., 1999; Abdel-Naser *et al*., 2005). Our previous lab results have demonstrated the increased levels of *TNFA*, *TNFB*, *IFNG*, and *IL4* in the circulation of vitiligo patients (Laddha *et al.,* 2012; Laddha *et al.,* 2013; Dwivedi *et al.,* 2013; Imran *et al.,* 2012).

Albeit, dissimilar outcomes are reported for few cytokines and candidate genes, with respect to its expression levels and genetic association with vitiligo, suggesting the involvement of other factors and role of ethnicity in disease precipitation (Figure 1). Thus, it is pertinent to study the levels of key candidate genes in the same set of skin samples. Overall, the above results indicate the importance of assessing the transcript levels of these cytokines in the microenvironment of skin of vitiligo patients. IFN-γ plays an important role in maintaining the homeostasis of melanocyte biology and its levels are important for maintaining cytokine balance. IFN-γ and IFN-γ-induced chemokines, specifically, CXCL10 and its cognate receptor C-X-C Motif Chemokine Receptor (CXCR)-3 are found to be increased in lesional skin and serum samples of patients with vitiligo, and also seen to be upregulated in autoreactive T cells in the blood and skin of patients with vitiligo (Rashighi *et al.,* 2014; Ezzedine *et al.,* 2015; Harris, 2015)**.** We have also found significantly increased transcript levels of *IFNG* in lesional as well as non-lesional skin of vitiligo patients (Figure 8). Increased levels of TNF-α have been reported in the lesional skin (Moretti *et al.,* 2002; Grimes *et al.,* 2004; Birol *et al.,* 2006) which are in accordance with our results, where we have found increased levels of *TNFA* in lesional and non-lesional skin of vitiligo patients (Figure 2). Interleukin 1 regulation is important for immune homeostasis whereas increased levels of IL-1α and IL-1β were reported in vitiligo (Birol *et al.,* 2006; Tu *et al.,* 2003; Dani *et al.,* 2017). Increased levels of IL-6 in skin (Moretti *et al.,* 2009) have also been reported in vitiligo patients. Unlike other studies, we have found decreased levels of *IL10* in skin samples of vitiligo patients (Figure 7). On the contrary, Grimes *et al.,* (2004) and Aydingoz *et al.,* (2015) have reported increased levels of *IL10* in skin and serum samples of vitiligo patients. After topical application of tacrolimus, Grimes *et al.,* (2004) found no significant difference in the *IL10* transcript levels, warranting the need for further investigation of genetic variants and levels of *IL10* in vitiligo patients.

It has been shown that human keratinocytes constitutively express inflammasome proteins together with pro-IL-1α and β and pro-IL-18 (Feldmeyer *et al.,* 2007). IL-1β activation in human epidermis can also occur *via.* an alternative mechanism involving stratum corneum chymotryptic enzyme (kallikrein 7), a serine protease specifically expressed in keratinizing squamous epithelia (Lundqvist *et al.,* 1997). Our results showed increased *IL1B* expression levels in non-lesional skin as compared to lesional skin (Figure 4), substantiating its important role in disease progression and are in accordance with studies of Wang *et al.,* (2011). They have also found increased level of *IL1B* in skin samples of vitiligo patients. IL-1β acts on lymphocytes and up regulates IL-2 receptor expression, prolonging the survival of T-cells, enhancing the antibody production by B-cells and increasing B-cell proliferation. IL-1β also helps in differentiation and proliferation of Th17 cells. Thus, IL-1β amplifies T and B-cell responses that might lead to destruction of melanocytes in the lesional skin (Marie *et al.,* 2014). Non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1* and *IL1RN* were observed in lesional and non-lesional skin of vitiligo patients compared to controls. A previous report showed that the IL-4 levels were decreased in peri-lesional skin, when compared to non-lesional and lesional skin (Wang *et al.,* 2011). However, we did not find difference in the expression levels of *IL4*. Overall, our studies provide evidence of cytokine imbalance in the microenvironment of skin of vitiligo patients (Table 3).

In conclusion, we have established the existence of epidermal cytokine imbalance between pro and anti–inflammatory cytokines in the skin microenvironment of vitiligo patients. We have found that *TNFA, IL1B, IFNG* and *IL10* are potential genes and might contribute to autoimmune mediated pathogenesis of vitiligo.

3.5 REFERENCES

- Abanmi, A., Al Harthi, F., Zouman, A., Kudwah, A., Jamal, M.A., Arfin, M. and Tariq, M., 2008. Association of Interleukin-10 gene promoter polymorphisms in Saudi patients with vitiligo. *Disease markers, 24*(1), pp.51-57.
- Abdel-Naser, M.B., 1999. Differential effects on melancyte growth and melanization of low vs. high calcium keratinocyte-conditioned medium. *British Journal of Dermatology, 140*(1), pp.50-55.

- Abdel-Naser, M.B., Abdallah, M., De Almeida, H.L. and Wollina, U., 2005. Human skin cell culture and its impact on dermatology. *Egyptian dermatology online journal*, *1*(2), p.2
- Alkhateeb, A. and Qarqaz, F., 2010. Genetic association of NALP1 with generalized vitiligo in Jordanian Arabs. *Archives of dermatological research*, *302*(8), pp.631-634.
- Aydıngöz, I.E., Kanmaz-Özer, M., Gedikbaşi, A., Vural, P., Doğru-Abbasoğlu, S. and Uysal, M., 2015. The combination of tumour necrosis factor-α− 308A and interleukin-10−1082G gene polymorphisms and increased serum levels of related cytokines: susceptibility to vitiligo. *Clinical and experimental dermatology*, *40*(1), pp.71-77
- Aydıngöz, I.E., Kanmaz-Özer, M., Gedikbaşi, A., Vural, P., Doğru-Abbasoğlu, S. and Uysal, M., 2015. The combination of tumour necrosis factor-α− 308A and interleukin-10− 1082G gene polymorphisms and increased serum levels of related cytokines: susceptibility to vitiligo.*Clinical and experimental dermatology*, *40*(1), pp.71-77.
- Begum, R., Marfatia, Y.S., Laddha, N.C., Dwivedi, M., Mansuri, M.S. and Singh, M., 2014. Vitiligo: a complex disease and a complex approach.*Molecularcytogenetics*, *7*(1), p.I57.
- Birol, A., Kisa, U., Kurtipek, G.S., Kara, F., Kocak, M., Erkek, E. and Caglayan, O., 2006. Increased tumor necrosis factor alpha (TNF-α) and interleukin 1 alpha (IL1-α) levels in the lesional skin of patients with nonsegmentalvitiligo. *International journal of dermatology*, *45*(8), pp.992-993.
- Dani, P., Patnaik, N., Singh, A., Jaiswal, A., Agrawal, B., Kumar, A.A., Varkhande, S.R., Sharma, A., Vaish, U., Ghosh, P. and Sharma, V.K., 2017. Association and expression of antigen processing gene PSMB8 coding for Low Molecular Mass Protease 7 (LMP7) with Vitiligo in North India: case-control study. *British Journal of Dermatology*.
- Dwivedi, M., Laddha, N.C., Shah, K., Shah, B.J. and Begum, R., 2013. Involvement of interferon-gamma genetic variants and intercellular adhesion

molecule-1 in onset and progression of generalized vitiligo. *Journal of Interferon & Cytokine Research*, *33*(11), pp.646-659.

- Ezzedine, K., Sheth, V., Rodrigues, M., Eleftheriadou, V., Harris, J.E., Hamzavi, I.H. and Pandya, A.G., 2015. Vitiligo is not a cosmetic disease. *Journal of the American Academy of Dermatology*, *73*(5), pp.883-885.
- Faul, F., Erdfelder, E., Lang, A.G. and Buchner, A., 2007. G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior research methods*, *39*(2), pp.175-191.
- Feldmeyer, L., Keller, M., Niklaus, G., Hohl, D., Werner, S. and Beer, H.D., 2007. The inflammasome mediates UVB-induced activation and secretion of interleukin-1β by keratinocytes. *Current Biology*, *17*(13), pp.1140-1145.
- Grimes, P.E., Morris, R., Avaniss-Aghajani, E., Soriano, T., Meraz, M. and Metzger, A., 2004. Topical tacrolimus therapy for vitiligo: therapeutic responses and skin messenger RNA expression of proinflammatory cytokines. *Journal of the American Academy of Dermatology*, *51*(1), pp.52-61.
- Harris, J.E., 2015. IFN-γ in Vitiligo, Is It the Fuel or the Fire?. *Actadermato-venereologica*, *95*(6), pp.643-644.
- Imran, M., Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, J., Rani, R., Gokhale, R.S., Sharma, V.K., Marfatia, Y.S. and Begum, R., 2012. Interleukin-4 genetic variants correlate with its transcript and protein levels in patients with vitiligo. *British Journal of Dermatology*, *167*(2), pp.314-323.
- Laddha, N.C., Dwivedi, M. and Begum, R., 2012. Increased Tumor Necrosis Factor (TNF)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo. *PLoS One*,*7*(12), p.e52298.
- Laddha, N.C., Dwivedi, M., Gani, A.R., Mansuri, M.S. and Begum, R., 2013. Tumor Necrosis Factor B (TNFB) genetic variants and its increased expression are associated with vitiligo susceptibility. *PloS one*, *8*(11), p.e81736.
- Laddha, N.C., Dwivedi, M., Mansuri, M.S., Gani, A.R., Ansarullah, M., Ramachandran, A.V., Dalai, S. and Begum, R., 2013. Vitiligo: interplay between

oxidative stress and immune system. *Experimental dermatology*, *22*(4), pp.245-250.

- Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, M., Gani, A.R., Yeola, A.P., Panchal, V.N., Khan, F., Dave, D.J., Patel, A. and Madhavan, S.E., 2014. Role of oxidative stress and autoimmunity in onset and progression of vitiligo. *Experimental dermatology*, *23*(5), pp.352-353.
- Laddha, N.C., Dwivedi, M., Mansuri, M.S., Singh, M., Patel, H.H., Agarwal, N., Shah, A.M. and Begum, R., 2014. Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility. *PloS one*, *9*(9), p.e107020.
- Lee, Y.S., Park, K.C., Youn, S.W., Bang, H.D. and Kim, K.H., 1995. Polymorphism in the IL-1 Receptor Antagonist Gene in Vitiligo. *Annals of Dermatology*, *7*(4), pp.299-302.
- Lundqvist, E.N. and Egelrud, T., 1997. Biologically active, alternatively processed interleukin-1β in psoriatic scales. *European journal of immunology*, *27*(9), pp.2165-2171.
- Mansuri, M.S., Singh, M., Jadeja, S.D., Gani, A.R. and Patel, R., 2014. Could ER stress be a major link between oxidative stress and autoimmunity in Vitiligo. *PigmentaryDisord*, *1*, p.123.
- Marie, J., Kovacs, D., Pain, C., Jouary, T., Cota, C., Vergier, B., Picardo, M., Taieb, A., Ezzedine, K. and Cario-André, M., 2014. Inflammasome activation and vitiligo/nonsegmental vitiligo progression. *British Journal of Dermatology*, *170*(4), pp.816-823.
- Moretti, S., Fabbri, P., Baroni, G., Berti, S., Bani, D., Berti, E., Nassini, R., Lotti, T. and Massi, D., 2009. Keratinocyte dysfunction in vitiligo epidermis: cytokine microenvironment and correlation to keratinocyte apoptosis.*Histology and histopathology*, *24*(7), p.849.
- Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M. and Fabbri, P., 2002. New insights into the pathogenesis of vitiligo: imbalance of

epidermal cytokines at sites of lesions. *Pigment Cell & Melanoma Research*, *15*(2), pp.87-92.

- Namian, A.M., Shahbaz, S., Salmanpoor, R., Namazi, M.R., Dehghani, F. and Kamali-Sarvestani, E., 2009. Association of interferon-gamma and tumor necrosis factor alpha polymorphisms with susceptibility to vitiligo in Iranian patients. *Archives of dermatological research*, *301*(1), pp.21-25.
- O'Shea, J.J., Ma, A. and Lipsky, P., 2002. Cytokines and autoimmunity. *Nature Reviews Immunology*, *2*(1), pp.37-45.
- Pehlivan, S., Ozkinay, F., Alper, S., Onay, H., Yuksel, E., Pehlivan, M. and Ozkinay, C., 2009. Association between IL4 (–590), ACE (I)/(D), CCR5 (Δ32), CTLA4 (+ 49) and IL1-RN (VNTR in intron 2) gene polymorphisms and vitiligo. *European Journal of Dermatology*, *19*(2), pp.126-128.
- Rashighi, M., Agarwal, P., Richmond, J.M., Harris, T.H., Dresser, K., Su, M.W., Zhou, Y., Deng, A., Hunter, C.A., Luster, A.D. and Harris, J.E., 2014. CXCL10 is critical for the progression and maintenance of depigmentation in a mouse model of vitiligo. *Science translational medicine*, *6*(223), pp.223ra23-223ra23.
- Salmon, J.K., Armstrong, C.A. and Ansel, J.C., 1994. The skin as an immune organ. *Western journal of medicine*, *160*(2), p.146.
- Slominski, A., Zbytek, B. and Slominski, R., 2009. Inhibitors of melanogenesis increase toxicity of cyclophosphamide and lymphocytes against melanoma cells. *International journal of cancer*, *124*(6), pp.1470-1477.
- Tam, I. and Stępień, K., 2007. Melanocytes–immunocompetent pigmented cells. *Advances in Dermatology and Allergology/PostępyDermatologii i Alergologii*, *24*(4), pp.188-193.
- Tu, C.X., Gu, J.S. and Lin, X.R., 2003. Increased interleukin-6 and granulocyte–macrophage clony stimulating factor levels in the sera of patients with non-segmental vitiligo. *Journal of dermatological science*,*31*(1), pp.73-78.
- Wang, C.Q., Cruz-Inigo, A.E., Fuentes-Duculan, J., Moussai, D., Gulati, N., Sullivan-Whalen, M., Gilleaudeau, P., Cohen, J.A. and Krueger, J.G., 2011. Th17

cells and activated dendritic cells are increased in vitiligolesions.*PloS one*, *6*(4), p.e18907.

- Yun, J.Y., Uhm, Y.K., Kim, H.J., Lim, S.H., Chung, J.H., Shin, M.K., Yim, S.V. and Lee, M.H., 2010. Transforming growth factor beta receptor II (TGFBR2) polymorphisms and the association with nonsegmental vitiligo in the Korean population. *International journal of immunogenetics*, *37*(4), pp.289-291.