

## Evaluation of the Role of Apoptosis in Vitiligo: Immunohistochemical Expression of P53, Bcl-2 and MART-1 Antigens

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### Abstract

It is clear that melanocytes are absent from the vitiliginous skin. However, the mechanism of melanocyte disappearance has never been clearly understood. It is not clear whether melanocyte loss in vitiligo results from apoptosis or other causes of cell death. A very few data were published about the role of apoptosis in vitiligo.

To investigate the role of apoptosis in vitiligo, ten biopsies from patients and ten normal control biopsies were taken from the skin of the thigh by the scalped biopsy technique. Each biopsy included adjacent areas of the lesional, peri-lesional and non-lesional skin. All subjects were males selected at Al-Housein University Hospital and ranged between 25 to 43 years in age.

The expression of MART-1 protein as a melanocyte marker, Bcl-2 as an antiapoptotic molecule and P53 as a proapoptotic molecule were evaluated by the immunoperoxidase technique and computerized image analysis. The study revealed complete absence of MART-1 positive melanocytes in the lesional skin, gradual appearance in the peri-lesional areas and showed no significant difference between the non-lesional areas and the normal skin ( $P=0.43$ ). The melanocytic Bcl-2 expression was higher in normal skin than the non-lesional area, lower in the peri-lesional areas and lowest in the lesional areas ( $p<0.05$ ). Conversely, more expression of melanocytic P53 was seen in the lesional than the peri-lesional areas, lower in the non-lesional skin and lowest in the normal skin ( $p<0.05$ ). Accordingly, the study revealed more susceptibility to apoptosis in the vitiliginous skin than in normal skin. We hope that these data can help in understanding the pathogenesis and the progression of this disease as well as providing better lines of its treatment.

**Key words: Apoptosis, Bcl-2, P53, Immunohistochemistry, Melanocytes, Vitiligo.**

### Introduction:

Vitiligo is an acquired progressive skin disorder characterized by depigmentation caused by selective destruction of melanocytes which are highly specialized cells responsible for skin pigmentation (*Le Poole et al., 1993*). Clinically, vitiligo is manifested as white macules on the skin that can be localized or generalized. The course of the disease is unpredictable but is often progressive with phases of stabilized depigmentation (*Castanet and Ortonne, 1997*).

Normally, the epidermis has a stratified, keratinizing epithelium consisting of keratinocytes (85-90%) and non-keratinocyte cells usually with a dendritic

morphology, representing 10-15% of the total cell population; these include Langerhans cells, melanocytes and Merkel cells. Melanocytes are cells with a dendritic morphology, of neural crest origin located within the basal cell layer. The ratio of melanocytes to basal keratinocytes along the dermoepidermal junction is site dependent and ranges from approximately 1:4 in the cheek to 1:11 in the thigh (*Breathnach, 1971*). They label on routinely-processed tissue sections with antibodies to MART-1 protein (*Kanitakis, 1998, Palermo et al, 2001 and Carlson et al., 2002*). Melanocytes also constitutively express bcl-2 oncoprotein; a 25 kDa cytoplasmic

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protein encoded by an oncogene preventing cells from undergoing apoptosis (*Van der Ord et al., 1994 and Kanitakis et al., 1995*), the c-kit protein (*Norris et al., 1996*) and tyrosinase. On the other hand, melanocytes and Langerhans cells are the only intraepidermal cells expressing vimentin (*Kanitakis, 1998*).

Numerous hypotheses about the etiology of vitiligo had been found however, none of them was proven. One way to understand the etiology was to determine the mechanism by which melanocytes were destroyed. Two known mechanisms for this cell loss were found, necrosis and apoptosis (*Huang et al, 2002*). The histology of vitiligo strongly suggests apoptosis rather than necrosis as the mechanism for melanocyte destruction (*Biossy et al, 1991 and Abdel- Naser et al, 1994*).

Apoptosis or programmed cell death is modulated by a set of genes within the cells and considered as an important mechanism for control the number and location of cells. The removal of the cells is performed in a highly controlled, a very precise and a discriminating manner as the by-products from the cell constituents are hidden from the immune system and do not initiate an immune response like in necrosis. In addition, apoptosis is initiated either within the cell or by outside signals (*Malmusi and Ackerman, 2000*).

Several proteins of the Bcl-2 family including Bcl-2 itself, Bcl-x1 and Bcl-w, act as antiapoptotic protecting the cell from destruction. On the other hand, the P53 proteins function as proapoptotic inducing the cell into apoptosis; these two sets of molecules antagonize and balance each other (*Huang et al., 2002*).

Certain factors may induce apoptosis in the epidermis either by suppressing expression of Bcl-2 and/or enhance the expression of P53 such as the ultraviolet light especially ultraviolet B (UVB) (*Okamoto et al, 1999 and Van Den Wijngaard et al, 2000*). It might be postulated that those individuals with genetically low susceptibility to sun-induced cancers, are protected via the P53 mechanism. They have a low threshold for

induction of apoptosis and are prone to vitiligo but resistant to skin cancers. That description fits, at least, a large population of vitiligo patients (*Leffell, 2000 and Mc Kay et al, 2000*).

The aim of the work is to study the role of apoptosis in vitiligo through investigating the levels of expression of Bcl-2 and P53 as they maintain the balance against or toward cell death. In this study, we compared the lesional skin to the perilesional, non-lesional and the normal skin as well.

### Subjects & Methods

#### The study was performed on two groups:

- Group-1: Included 10 vitiligo patients (generalized vitiligo vulgaris).
- Group-2: Included 10 normal control subjects.

Cases of group-1 were selected at the Department of Dermatology, while cases of group-2 were selected at the Department of Plastic Surgery from patients attending for cosmetic problems, at Al-Housein University Hospital with consent.

Each biopsy was taken by the scalped biopsy technique from the skin of the thigh including lesional, peri-lesional and non-lesional areas. All cases ranged between 25 to 43 years in age. Specimens were formalin-fixed, paraffin-embedded and cut at 6 µm and subjected to the following techniques:

#### 1) Haematoxylin and Eosin (H&E) staining:

The staining was done to explore the morphologic appearance of the skin and to ensure the integrity of the specimens before the immunoperoxidase technique.

#### 2) The immunoperoxidase (IP) technique:

According to the manufacturer instructions of the UltraVision Large Volume Detection System (Lab-Vision CA. USA, Catalog Number TP-060-HL), immunoperoxidase method for detection of MART-1 protein as a specific melanocyte marker, Bcl-2 as an anti-apoptotic protein and P53 as a pro-apoptotic protein were done.

Paraffin sections were downed to water. The endogenous peroxidase activity was blocked by incubating the sections in hydrogen

peroxide for 10 minutes and washed twice in phosphate buffered saline (PBS). Enzyme digestion was performed by incubating the sections in 0.1% trypsin solution at 37°C for 10 minutes followed by 4 changes in PBS. To block nonspecific background staining,

sections were incubated in the “Ultra V Block” for 5 minutes at room temperature followed by rinsing in PBS. The sections were incubated in the primary monoclonal antibody according to table 1:

**Table 1: Types and application of monoclonal antibodies**

Antibody	Company	Catalog No	Incubation
MART-1	Zymed Lab.	18-7238	overnight
Bcl-2	NeoMarkers	MS-123-R7	overnight
P53	Dako	M7001	overnight

The sections were washed 4 times in PBS, then, incubated in the biotinylated Goat Anti-Polyvalent solution for 10 minutes at room temperature followed by 4 changes in PBS. The streptavidin peroxidase was applied for 10 minutes at room temperature, then sections were washed 4 times in PBS. Finally the sections were incubated with the chromogen substrate for 10 minutes, counterstained with haematoxylin and coverslipped.

**Quantitative Analysis**

All sections were examined after staining by the computerized image analysis. The mean percentage of positive areas to the total epidermal areas was calculated using Image Pro Plus V4.51 (Media Cybernetics Inc. 2002). The statistical analysis was carried out according to the standard statistical procedures and Student (t) test to estimate the significance of results using Microsoft Excel XP 2002.

**Results**

**Morphological Examination:**

Haematoxylin and eosin (H&E) staining revealed absolute loss of melanocytes in the lesional areas confirmed by the immunohistochemistry of MART-1. There was more availability of melanocytes in the non-lesional than in perilesional

areas. The epidermis of the lesional areas showed also a relative lack of regular arrangement and cell distribution in the epidermis than the non-lesional areas (Fig. 4).

**Molecular Expression:**

Microscopic examination revealed complete absence of MART-1 positive melanocytes in the lesional skin, and more availability in the non-lesional than in perilesional areas. The later showed gradual loss of positive cells toward the lesional area (P <0.05). There was no significant difference between the normal control and the non-lesional skin (P=0.43) (Table 2, Fig. 1& 5).

The melanocytic Bcl-2 expression was higher in normal skin than the non-lesional area, lower in the peri-lesional areas and lowest in the lesional areas (p<0.05). The peri-lesional areas showed gradual loss of positive cells toward the lesional area (Table 3, Fig. 2& 6).

Conversely, more expression of melanocytic P53 was seen in the lesional than the peri-lesional areas, lower in the non-lesional skin and lowest in the normal skin (p<0.05). The peri-lesional areas showed gradual increase of positive cells toward the lesional area (Table4, Fig. 3& 7).

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**Table 2: The Mean Percent of MART-1 Positive area to the total Epidermal area**

	1	2	3	4		
	Cont	N-L	Peri-L	Lesional		
Average	4.422	4.309	2.78	0		
SD	0.27	0.42	0.19	0		
Gr-Comp	1-2	2-3	1-3	1-4	3-4	2-4
P-Value	0.43	0.009	0.011	< 0.00001	< 0.00001	<0.00001

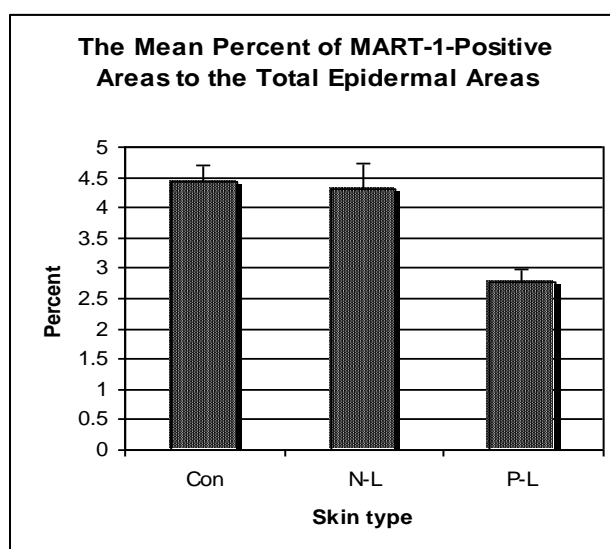
**Table 3: The Mean Percent of BCL2 Positive area to the total Epidermal area**

	1	2	3	4		
	Cont	N-L	Peri-L	Lesional		
Average	4.035	3.8	2.085	1.042		
SD	0.19	0.21	0.13	0.16		
Gr-Comp	1-2	2-3	1-3	1-4	3-4	2-4
P-Value	0.00015	0.0006	0.007	0.00014	0.0011	0.003

**Table 4: The Mean Percent of P53 Positive area to the total Epidermal area**

	1	2	3	4		
	Cont	N-L	Peri-L	Lesional		
Average	0.095	0.108	0.329	0.422		
SD	0.0002	0.001	0.001	0.0007		
Gr-Comp	1-2	2-3	1-3	1-4	3-4	2-4
P-Value	0.08	0.0011	0.0003	0.000001	0.0006	0.00013

*Gr-Comp= groups compared, Con= control, N-L= non-lesional, P-L= peri-lesional, L= lesional, SD= standard deviation*



**Figure 1**

*The lesional skin was not included in the graph since it was quite negative to the MART-1*

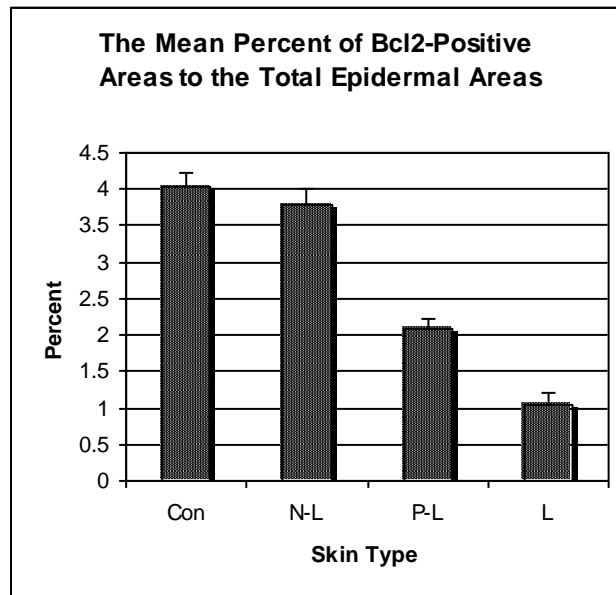


Figure 2

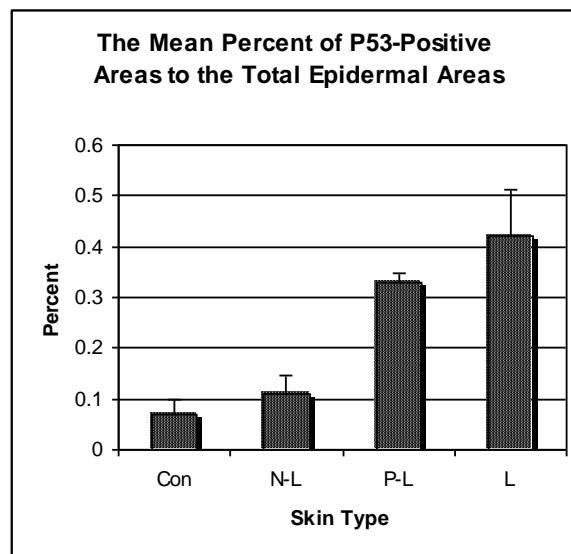
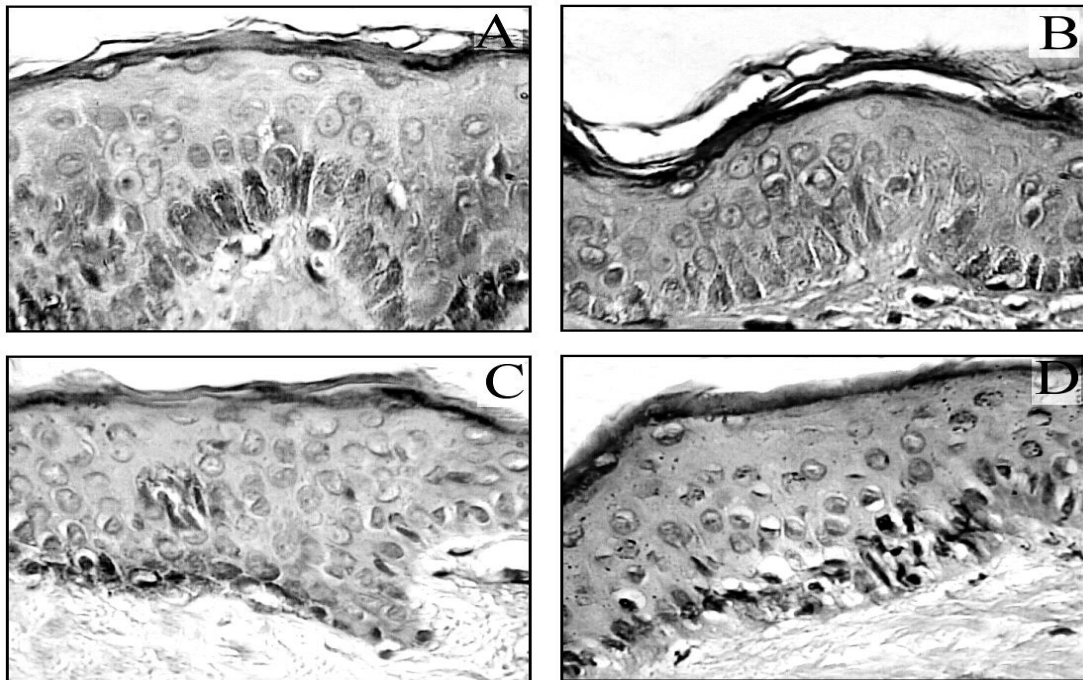


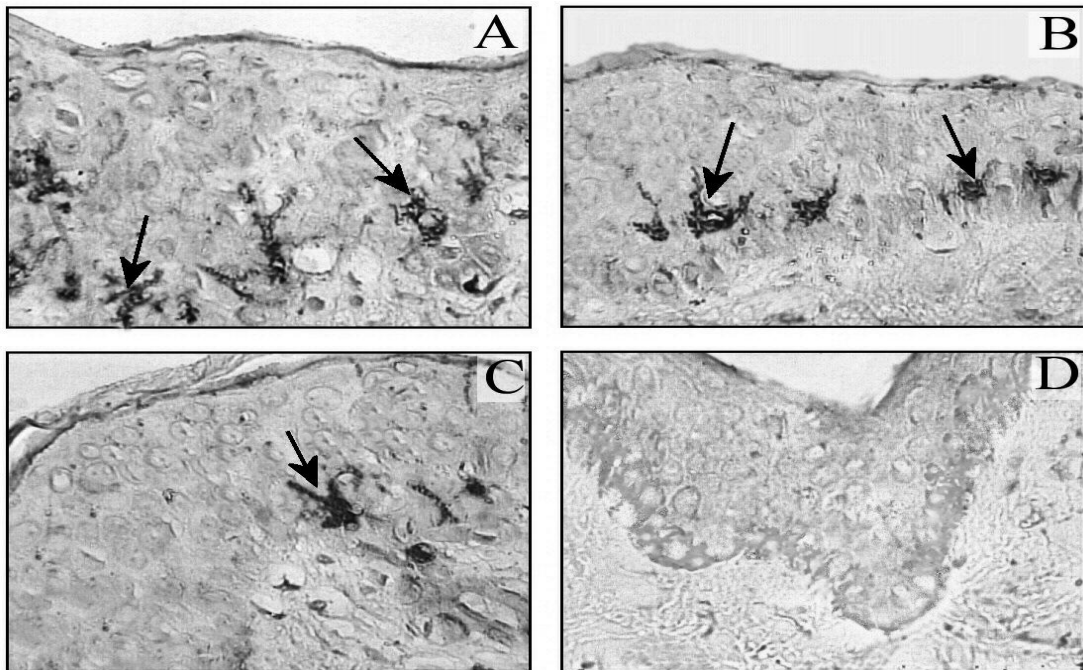
Figure 3

*Con= control, N-L= non-lesional, P-L= peri-lesional, L= lesional*  
*Error bars represent the standard deviation (SD).*



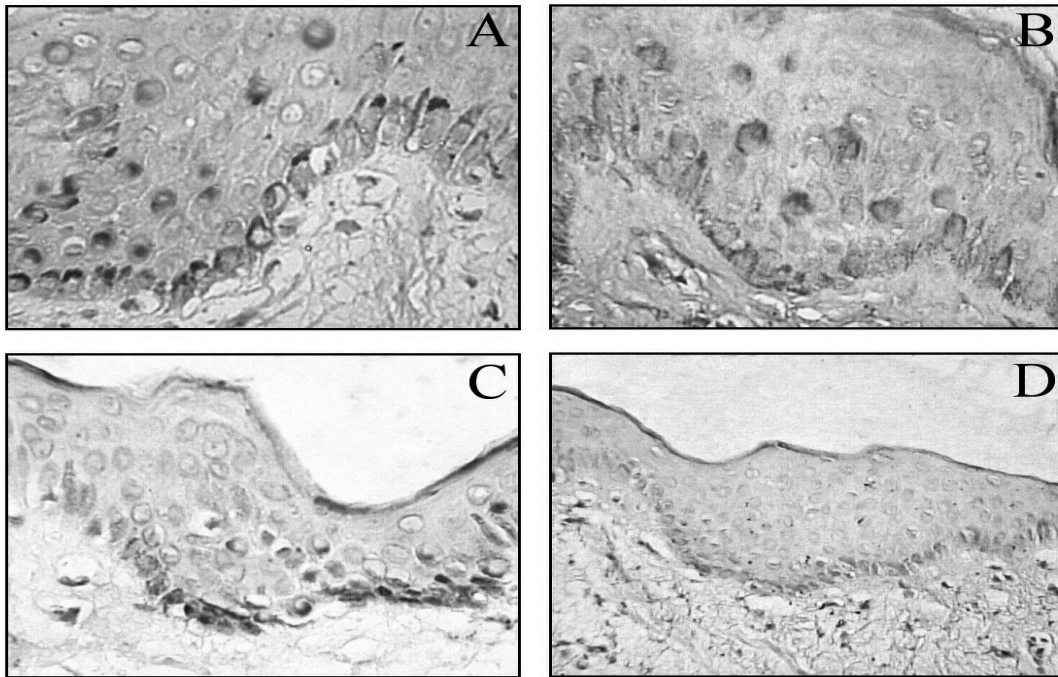
**Figure (4)**

*The Morphological appearance of the skin after H&E staining. In normal skin (A), melanocytes are present and the epidermal cells are organized. In the non-lesional skin (B), the epidermis is relatively similar to the control. In the peri-lesional area there is gradual loss of melanocytes (C) which are totally lost in the lesional area with disorganization of the epidermal layers (D). (H&E, x400)*



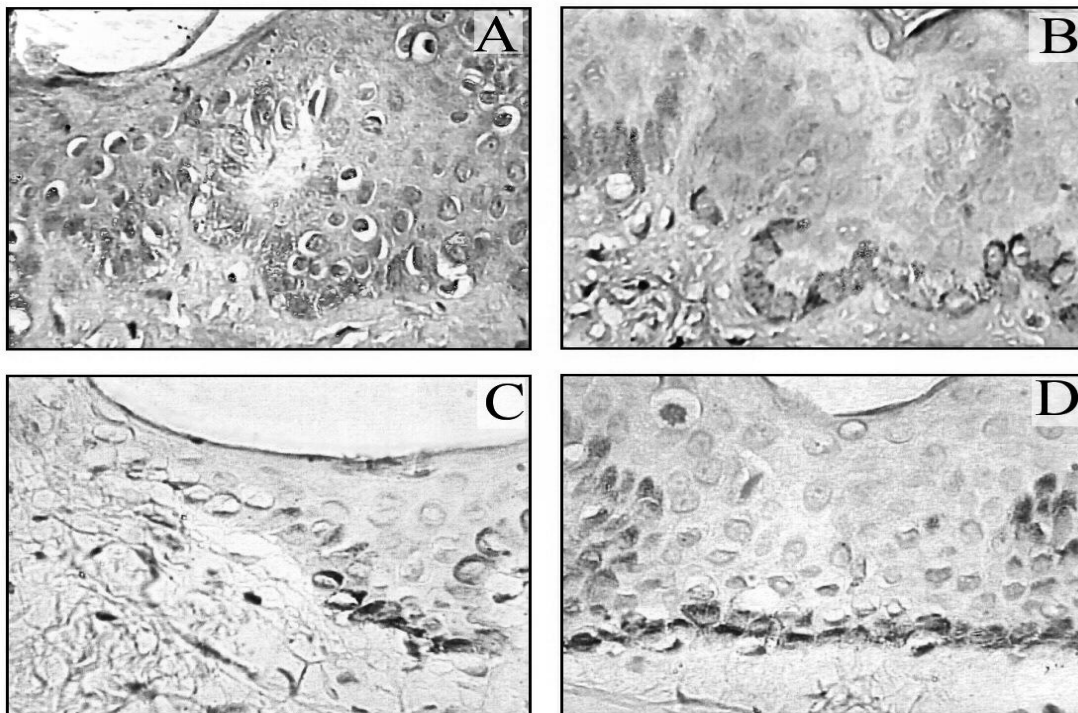
**Figure (5)**

*Immunohistochemical labeling of melanocyte with MART-1 showing normal pattern in the control (A) and similar picture in the non-lesional skin (B), gradual loss in the peri-lesional area (C) and total absence in the lesional skin (D). (Immunoperoxidase (IP) x 400).*



**Figure (6)**

*The expression of Bcl-2 protein showing a relatively higher level of expression in the control (A) and slightly lower level in the non-lesional skin (B), gradual loss in the peri-lesional area (C) and maximal decrease in the lesional skin (D) (IP X 400).*



**Figure (7)**

*The expression of P-53 protein showing lowest levels of expression in the control (A) and slightly higher level in the non-lesional skin (B), gradual increase in the peri-lesional area (C) and the highest levels in the lesional skin (D). (IP X 400).*

## Discussion:

Vitiligo is a skin disease characterized by white spots that usually progress with enlargement of the existing lesions and appearance of new ones. The cause is the selective destruction of melanocytes that are absent in established lesions. In the normal skin, melanocytes are regularly and predictably arrayed in the epidermis along the dermoepidermal junction ( *Breathnach, 1971*).

The melanocyte-specific melanosomal-related protein, Mart-1 (Melan-A) is an antigen recognized by autologous cytotoxic T cells (*Kawakami et al, 1994 & 1997 and Chen et al, 1996*). Our study showed complete absence of MART-1 positive melanocytes in the lesional skin coinciding with that reported by *Le Poole et al, (1993)* and *Castanet & Ortonne 1997*; who also stated that the melanocytic loss is thought to be due to their destruction in-situ.

*Carlson et al, 2002* suggested that there are many melanocytes (melanogenic and nonmelanogenic) within the epidermis that cannot be detected by standard methods. Some of these Mart-1-labeled melanocytes may represent the so-called precursor melanocytes that were detected by *Grichnik et al, 1996*. The absence of melanocytes demonstrated in most studies of vitiligo may be partially due to searching for functioning melanocytes with active melanogenesis (*Le Poole et al, 1993; Bhawan, 1997; Norris et al, 1996; and Hann et al, 2000*). Inactive melanocytes may exist in vitiligo that awaits the necessary signals or environmental change to begin the normal melanogenesis (*Carlson et al, 2002*).

As an acute inflammatory response is not a general finding in vitiligo (*Le Poole et al, 1996*), it was hypothesized that melanocytes die in a controlled manner via apoptosis (*Cohen, 1993*). Because melanocytes in patients with vitiligo may intrinsically be more susceptible to apoptotic stimuli, we compared melanocytes from control individuals to those of vitiligo patients through expression of Bcl-2 and P53. Apoptosis is induced when the anti-apoptotic factors are overcome by

lethal stimuli or lose their natural defenses. Newer reports stated that the melanocytic cell death in vitiligo, particularly the generalized type, occurred in the absence of neutrophil influx and erythema, suggesting apoptosis (*Rudin & Thompson 1997 and Huang et al, 2002*).

Bcl-2 was investigated because of its important opposing role in regulating the susceptibility to apoptosis (*Van Den Wijngaard et al, 2000*). In accordance with earlier reports (*Plettenberg et al, 1995; Saenz-Santamaria et al, 1994*), in-situ analysis revealed high melanocytic bcl-2 expression in normal skin. Equally, high bcl-2 expression was observed in the non-lesional skin (*Van Den Wijngaard et al, 2000*). In this work, we have detected a comparatively higher value for bcl-2 expression in the normal control skin than other investigated regions.

The expression of bcl-2 can be regulated by the tumor suppressor gene P53 (*Miyashita et al, 1994*) and it is known that P53 is involved in inducing apoptosis. Our study showed that P53 - expression was higher in the lesional and perilesional sites than in the control specimens. Moreover, the mean percent of melanocytic P53 positive areas showed a significant increase in the lesional skin compared to other areas ( $P < 0.05$ ), indicating more susceptibility to apoptosis. These findings are identical to those observed by *Campbell et al, 1993*, who reported an increase in the p53 expression, in the human skin after exposure to ultraviolet (UV) rays. It was also shown that UV is capable of modulating melanocyte-expressed apoptosis regulatory molecules leading to loss of melanocytes (*Van Den Wijngaard et al, 2000*).

It was reported that infiltrating T-cells and macrophages can be observed adjacent to the remaining peri-lesional melanocytes in generalized vitiligo and it was shown that cytotoxic T-cells, macrophages are both capable of inducing apoptosis of melanocytes in vitro. Therefore, it is feasible that in vivo melanocyte apoptosis in vitiligo is caused by auto-reactive T-cell and/or



macrophages (*Van Den Wijngaard et al, 2000*). However, apoptosis might be an alternative way to view the role of immune system and its involvement in melanocyte destruction.

### Conclusion

Our data strongly supported apoptosis rather than necrosis as a mechanism for the elimination of melanocytes in vitiligo. Therefore, the factors that can tilt the balance away from the pro-toward the anti-apoptotic control may prevent the spread of the disease. Accordingly, the drugs that can enhance the anti-apoptotic proteins e.g. bcl-2 or suppress the expression of proapoptotic ones e.g. P53 may provide a raical solution of this disorder.

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## تقييم دور التساقط الخلوى فى مرض البهاق : دراسة بالكيمياء النسيجية المناعية للظهور الجزئى لكل من بسبإل2، بى53 ومارت1

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من الواضح أن خلايا الميلانين تختفى من الجلد المصاب بالبهاق، ولكن الطريقة التى تختفى بها لم يتم فهمها حتى الآن، فليس من الواضح إن كانت تموت بالتساقط الخلوى أو بسبب آخر غير ذلك، وحتى الوقت الحاضر لم ينشر إلا عدد قليل جداً من الأبحاث فى هذا المجال.

لتقييم دور التساقط الخلوى فى مرض البهاق تم أخذ 10 عينات من الذكور المصابين بمرض البهاق و10 عينات ضابطة لأشخاص أصحاء من جلد الفخذ بحيث اشتملت كل عينة على كل من المنطقة المصابة والسليمة والمنطقة المتوسطة بينهما، وقد تم اختيار جميع الحالات بمستشفى الحسين الجامعى بحيث كانوا من الذكور وتراوحت أعمارهم بين 25-43 سنة.

وقد تم تقييم ظهور جزئيات مارت-1 (MART-1) و بى53 (p53) و بسبإل-2 (Bcl-2) بتقنية البيروكسيدىز المناعى ثم فحصت بتحليل الصور بالحاسب الآلى، وقد تأكد من الدراسة عدم وجود خلايا الميلانين فى الجلد المصاب بالمقارنة بظهورها تدريجياً فى المنطقة المتوسطة ثم بشكل طبيعى فى المناطق الغير مصابة التى لم يختلف الظهور فيه إحصائياً عن الجلد الطبيعى (قيمة ب الإحصائية = 0,43) ، كذلك ظهور جزئىء بسبإل-2 بصورة أكبر فى الجلد الطبيعى مقارنة بالمنطقة الغير مصابة ثم بصورة أقل فى المنطقة المتوسطة ثم أقل فى المنطقة المصابة (قيمة ب الإحصائية أقل من 0,05) ، وعلى العكس ظهر جزئىء بى53 بصورة أكبر فى المنطقة المصابة مقارنة بالمنطقة المتوسطة ثم بصورة أقل فى المنطقة الغير مصابة ثم أقل الجلد الطبيعى (قيمة ب الإحصائية أقل من 0,05).

وهكذا توضح هذه الدراسة زيادة تعرض خلايا الجلد المصاب للتساقط الخلوى أكثر من الجلد الطبيعى، كما نأمل أن تساهم هذه النتائج فى فهم طبيعة وتطور هذا المرض وإيجاد أساليب علاجية أفضل فى المستقبل القريب.