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ORIGINAL ARTICLE PEDIATRICS

Histopathological and molecular analysis in dermis and epidermis of patients with systemic and localized scleroderma

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ABSTRACT

OBJECTIVE: Scleroderma has a wide range of clinical manifestations due to vasculopathy, autoimmunity, altered endothelium function, and abnormal fibrosis, which are accused in the pathogenesis of the disease. The aim of this study is to shed light on the pathogenesis of the disease in childhood via dermal immunohistochemical analysis of the cases.

METHODS: A single-blind clinical trial is conducted with evaluation of the tissue samples obtained from patients. The samples are stained with PAS, hematoxylin and eosin, E-Cadherin, Connective tissue growth factor (CTGF), Tunnel, and staining for Transforming growth factor beta 1 (TGF-β1) and evaluated by light microscopy. In addition, both TGF-β1 level and mRNA expression analyses in plasma and tissue samples from patients are performed. A total of 15 patients (systemic, n=8 or localized; n=7) were enrolled in the study.

RESULTS: The mean age of onset of the disease was 9.2±1.2 years, and the mean age of diagnosis was 15.3±3.2 years. Antinuclear antibody (ANA) titer was between 1/160–1/640 in all patients with systemic sclerosis. There was no ANA positivity in patients with localized scleroderma. A total of 22 tissue samples (15 diseased tissues, 7 healthy tissues) were examined. Histopathological examination has shown that two clinically different subgroups have different characteristics at the tissue level.

CONCLUSION: TGF-β1 levels, which play a fundamental role in the pathogenesis of the disease, are found in both plasma and skin have been shown high. This elevation was found particularly in patients with systemic scleroderma to be more pronounced. Also, in patients with localized scleroderma, skin fibroblasts have been shown to limit the pathologic response.

Keywords: Childhood; scleroderma; transforming growth factor beta 1.

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Scleroderma is an autoimmune disease primarily affecting the skin and lungs, presenting major challenges in diagnosis and treatment. It falls into two main categories: systemic sclerosis (SSc) and localized scleroderma [1]. Both types are marked by abnormal skin fibrosis, characterized by an excessive buildup of extracellular matrix (ECM), a process driven largely by myofibroblasts [2]. This pathological phenomenon is less understood in pediatric cases, where it manifests with unique clinical features.

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Both systemic and localized forms of scleroderma are uncommon in children, comprising less than 10% of cases [3–5]. The disease affects both genders equally in early childhood, but after the age of 8, it becomes three times more common in females [6]. The disease can lead to fibrotic changes in organs such as the esophagus, lungs, gastrointestinal tract, and heart. Localized scleroderma varies from mild superficial morphea plaques to more severe lesions causing joint contractures and extremity asymmetry [7]. In both children and adults, the linear form is the most commonly observed localized variant [6, 8].

Several studies indicate that cytokines and growth factors like TGF-β and platelet-derived growth factor (PDGF) contribute to scleroderma's pathogenesis by increasing collagen and fibroblast synthesis [9, 10]. This suggests that T lymphocyte-associated autoimmunity influences tissue cytokines, leading to increased collagen production [11–14]. Immunological changes in scleroderma include the presence of serum autoantibodies against a range of antigens, T-cell infiltration, and intense mononuclear cell accumulation in early skin lesions. Further evidence of T cell hyperactivity in scleroderma is seen in elevated CD4+ cell levels and an increased CD4+/CD8+ cell ratio [15].

Also One of the significant consequences of vascular injury is the formation of anti-endothelial cell antibodies (AECAs). These antibodies play a dual role in the progression of scleroderma. Firstly, they directly contribute to microvascular damage, exacerbating the already compromised vascular integrity. Secondly, they indirectly influence the disease by activating endothelial cell adhesion molecule expression, which further stimulates endothelial damage. This process is crucial as it leads to enhanced endothelial apoptosis, marking a key event in the disease's progression [16].

Moreover, the role of Endothelin-1 (ET-1) in scleroderma cannot be overlooked. Originating from the endothelium, ET-1 is a potent vasoconstrictor agent. Its significance extends beyond mere vascular constriction; ET-1 also stimulates collagen synthesis [17]. It does so by increasing the activation of CTGF [18, 19]. This interplay between ET-1 and CTGF underscores a critical pathophysiological mechanism in scleroderma, where vascular dysfunction dovetails with the aberrant connective tissue synthesis, illustrating the multifaceted nature of the disease [19].

Recent research into scleroderma has highlighted the unique behavior of fibroblasts in the disease. These cells show resistance to Fas-dependent apoptosis, suggesting an evasion from the normal programmed cell death process. This resistance may contribute significantly to the pathological accumulation of fibrous tissue seen in scleroderma [9, 20].

Highlight key points

- The cutaneous expression patterns of E-Cadherin, CTGF, and TGF-ß in systemic sclerosis elucidated.
- The importance of TGF-β elevation in plasma and skin was emphasized, in the pathogenesis of both localized and systemic scleroderma.
- We predict that the results we obtained in this study will shed light on new treatment possibilities for scleroderma, the treatment of which is still controversial.

Fibroblasts are essential for maintaining organ structure and function. In the context of scleroderma, activated fibroblasts produce excessive amounts of collagen, leading to fibrosis and subsequent organ dysfunction. This excessive collagen production is central to the fibrotic characteristic of the disease and represents a key target for therapeutic intervention [21, 22].

Apoptosis and abnormal cell death processes are significantly implicated in scleroderma's pathogenesis. The disease involves critical pathways and proteins, such as Fas and Bax, which regulate these processes. The dysregulation of apoptosis contributes to the pathological changes observed in scleroderma [23–26].

E-Cadherin, an essential epithelial cadherin, plays a vital role in cell adhesion and differentiation. Its influence on cell shape and involvement in the structure of various epithelial layers are critical in the context of scleroderma. Disruptions in E-Cadherin function can lead to significant alterations in tissue architecture and disease progression [27].

Transforming Growth Factor-β (TGF-β) is a key regulator of immune cell functions. It modulates the proliferation and activation of these cells, playing a role in suppressing inflammatory and immune responses. TGF-β also promotes the release of IgA from B cells, illustrating its complex role in immune regulation and its relevance in scleroderma [28]. TGF-β1, a significant polypeptide, is involved in numerous cellular processes, including growth, proliferation, differentiation, and apoptosis. Its role is particularly crucial in the context of uncontrolled fibrosis in scleroderma and other diseases, where it contributes to the pathological accumulation of connective tissue [29].

The Connective Tissue Growth Factor (CTGF) is central in controlling the synthesis and breakdown of the extracellular matrix. It regulates matrix metalloproteinases and is significantly overexpressed in vascular alterations and fibrosis in systemic sclerosis. Understanding its role provides insights into the molecular mechanisms driving fibrosis [30].

Despite ongoing research, the exact origins of scleroderma remain elusive. It likely involves complex interactions involving changes in apoptosis, adhesion molecules, and cell connections in the skin. This study aims to investigate these aspects in-depth to better understand the pathogenesis of both localized scleroderma and systemic sclerosis, potentially leading to more effective treatment strategies.

MATERIALS AND METHODS

This single-blind clinical study was approved by the Ege University Faculty of Medicine Clinical Research Ethics Committee (number: B.30.2.EGE.0.20.05.00/OY/1131/498, date: 03.07.2012).

Informed written consent was taken from patients and their parents. A total of 15 patients were enrolled in the study. Skin samples were obtained from both the affected and healthy areas of localized scleroderma patients (n=7); and also from affected areas of patients with systemic sclerosis (n=8). Additionally, plasma samples from all patients and healthy donors were collected (n=12).

Histologic and Immuno-histochemical Procedures

Tissues underwent fixation through an overnight immersion in 4% paraformaldehyde (Merck), followed by dehydration, paraffin embedding, and sectioning at a 5µm thickness using the Leica RM 2145. Subsequent staining utilized standard Hematoxylin and Eosin (HE) as well as Periodic Acid Schiff (PAS) protocols.

Immunohistochemical Analyses

For immunohistochemical analyses, sections measuring 2 µm in thickness were utilized, featuring primary antibodies (e-Cadherin, CTGF, and TGF-β1), all diluted at 1:150. The deparaffinization process involved a one-hour immersion in xylene, followed by sequential rehydration in descending alcohol series, each lasting 2 minutes. After a 5-minute immersion in distilled water, tissues were positioned on object slides, washed in Phosphate Buffered Saline (PBS) for 10 minutes, and then treated with trypsin for 15 minutes. Subsequently, the primary antibody was applied in a 4°C incubator. The application of the biotinylated secondary antibody, PBS washing, and incubation with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride (DAB) ensued. Sections were counterstained with Mayer's Hematoxylin (Zymed Laboratories, Fisher Scientific) and mounted with Entellan.

The in situ detection of apoptosis at the single-cell level employed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) with the In Situ Cell Death Detection Kit, POD, Roche.

All sections underwent examination and photography using the Olympus C-5050 digital camera integrated with the Olympus BX51 light microscope. Blinded specialized investigators (HA, EEP, and FO) assessed group distinctions in the specimens, capturing five images from 10 different sections. The immunohistochemical staining intensity was semi-quantitatively graded based on the nuclear and cytoplasmic immunoreaction of the skin sections, categorized as follows: (−) no immunostaining, (+) weak staining, (++) moderate staining, and (+++) strong staining.

TGF-ß1 mRNA Isolation from Epidermis and Plasma

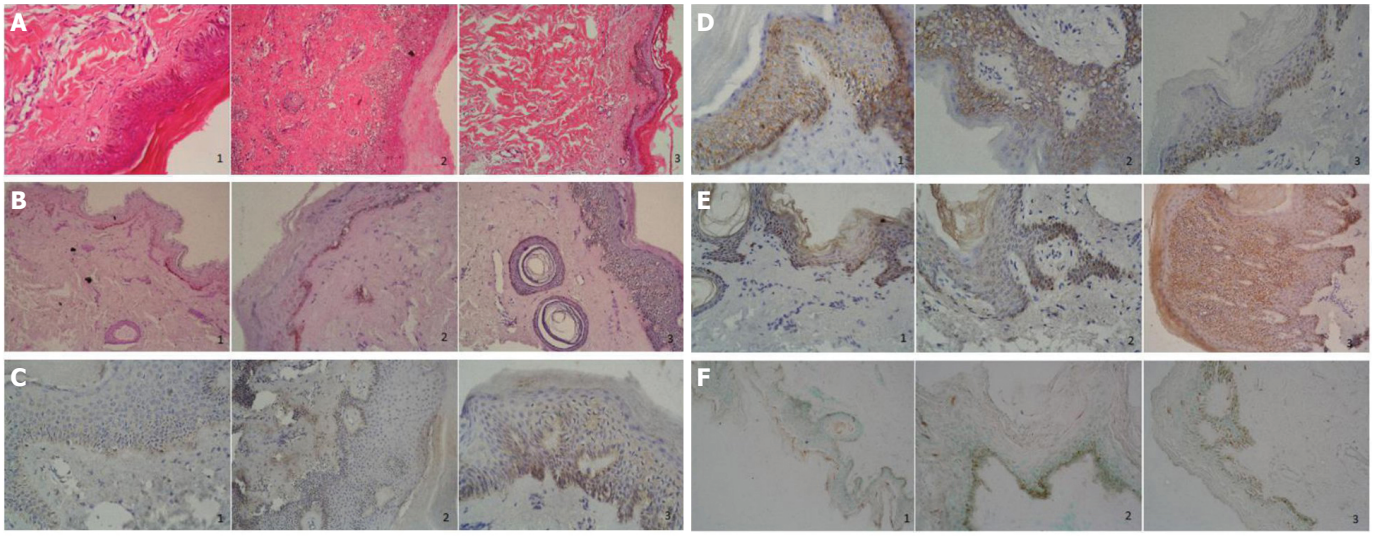
The extraction of total RNA from both skin biopsy specimens and plasma was carried out using the Magna Pure Compact RNA isolation kit. Subsequently, first-strand cDNA synthesis and quantitative real-time PCR were conducted utilizing the Transcriptor High Fidelity cDNA synthesis kit. The primer sets for TGF-ß1 and GAPDH were procured from the universal probe provided by Roche.

Analysis of Enzyme Levels TGFß1

Analysis of enzyme levels TGFß1 from plasma samples in patients and controls were obtained with Transforming Growth Factor beta 1, Human (TGFß1) Bio-Assay™ ELISA Kit.

Statistical Analysis

Statistical analyses were provided with the IBM SPSS (Statistics Package for Social Sciences for Windows, Version 22.0, Armonk, NY, IBM Corp.) package program. Quantitative data were expressed as mean ± SD and median (range). Qualitative data were expressed as absolute frequencies (number) and relative frequencies (percent). Depending on the normal distribution of the tested variable, analyses were performed using X2 test for categorical data and analysis of variance (ANOVA) or Mann-Whitney U test for continuous data. All tests were two-sided. p-value <0.05 was considered statistically significant.



Results of histopathological and immunhistochemical analyses of tissue samples in all patient groups			
	Localised scleroderma	**SSc**	**Control**
Histopathology			
Hematoxylene/Eosin (A)	Diffuse inflamation Sclerosis in papillary dermis Irregularity in Str. basale and Str. spinosum of epidermis Vacuolization in all layers	Sclerosis in papillary dermis (less than the localised form) Irregularity and distinct epidermal thinning in Str. spinosum Vacuolization in all layers	Normal
Periodic Acid Schiff (B)	Patchy lack of tissue in basement membrane	Tissue loss in basement membrane (more diffuse than the localised form)	Intact basement membrane
Immunohistochemistry			
Retention of CTGF (C)	Str.basale (++) Dermis(+)	Str.basale (++), Str. spinosum (++) Dermis(-)	Str.basale(+) Dermis(-)
e-Cadherin (D)	Str.basale (+) Str. spinosum(+)	Str.basale (+) Str. spinosum (-)	Str.basale (+++) Str. spinosum (+++)
TGF-ß1 (E)	Str.basale (++) Str. spinosum(+)	Str.basale (++), Str. spinosum (++)	Str.basale (+)
Tunnel staining (F)	Str.basale (+++) Str. spinosum(+++)	Str.basale (+++) Str. spinosum (+++)	Str.basale (+) Str. spinosum (-)

CTGF: Connective tissue growth factor SSc: Systemic sclerosis Str.: Stratum TGF-β1: Transforming growth factor beta 1

Figure 1. Hematoxylene / Eosin, Periodic acid –Schiff (PAS), Connective tissue growth factor, E-cadherin, Transforming growth factor beta (TGF-ß) and Tunnel staining of tissues. **(A)** Hematoxylene / Eosin staining of control (1), localized scleroderma (2) and systemic sclerosis (3). **(B)** Periodic Acid Schiff staining of control (1), localized scleroderma (2) and systemic sclerosis (3). **(C)** Retention of Connective tissue growth factor in samples of control (1), localized scleroderma (2) and systemic sclerosis (3). **(D)** E-cadherin staining of control (1), localized scleroderma (2) and systemic sclerosis (3). **(E)** Transforming growth factor beta staining of control (1), localized scleroderma (2) and systemic sclerosis (3). **(F)** Tunnel staining of control (1), localized scleroderma (2) and systemic sclerosis (3).

RESULTS

The study comprised a total of 15 children. Eight were diagnosed with SSc and 7 patients with localized scleroderma. Eleven of these children were female and 4 were male, with an age range of 9.2±1.2 years at the beginning of the disease. But their mean age at the time of diagnosis was 15.3±3.2 years.

Skin lesions were located in 5 patients in upper or lower extremity and in other two they were on abdominal skin.

All patients diagnosed with SSc had ANA positivity with a titer range of 1/160-1/640 whereas none of the localized scleroderma patients had any positive result. Results of histopathological and immunohistochemical analyses of tissue samples in all patient groups are shown in Figure 1.

Besides the pathological analysis of tissues, the measurement of TGF-ß1 enzyme levels in plasma specimens of all patients (n=15) and controls (n=12) showed a significant elevation in patient group (926±603.2 ng/ml) when compared to the control group (370±270 ng/ml; p=0.017) (Fig. 2). Additionally, the expression levels of TGF-ß1 RNA were measured in both blood and tissue specimens of both groups. Patient group had a mean plasma level of TGF-ß1 19.26±17.1 ng/ml; whereas the

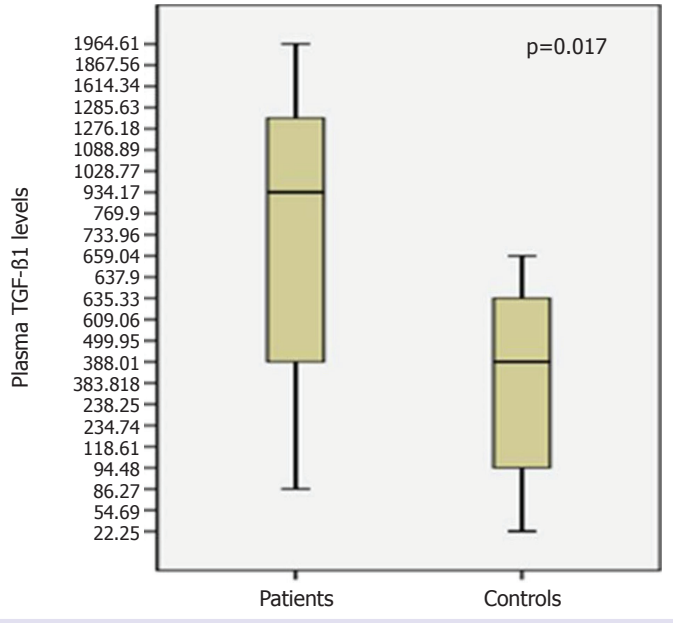


FIGURE 2. The plasma transforming growth factor beta levels in patients and controls.

control group had a mean level of TGF-ß1 37.7±13.8 ng/nl with a significant difference (p=0.006). This discrepancy was also observed in tissue analysis. The assessed tissue RNA expression levels exhibited a notable increase in the systemic group as opposed to both the localized and control groups of patients (Table 1).

TABLE 1. TGF-ß1 RNA expression levels in tissue specimens

	Mean	SD (±)
Localized group	2.93	2.08
Systemic group	3.73	1.5
Healthy tissue	0.36	0.21

p1 (all groups)=0.002; p2 (localized and systemic)=0.43; TGF-ß1: Transforming growth factor beta 1.

DISCUSSION

The outcomes of this study revealed the presence of diffuse inflammatory manifestations in the tissue examination of individuals diagnosed with localized scleroderma. Examination of the tissue showcased irregularities in the stratum basale and spinosum, along with indications of cell vacuolization across all epidermal layers. Sclerotic regions were identified in the papillary dermis. Conversely, in the tissue analysis of systemic sclerosis patients, sclerotic regions and inflammation were noted in the papillary dermis to a lesser extent compared to the localized type. Layered irregularities and thinning of the stratum spinosum are evident. Vacuolization findings in cells in all layers of the epidermis were also observed in this group (Fig. 1A). In the analysis performed with PAS dye, localized losses in the integrity of the basement membrane were found in the tissue taken from patients with localized scleroderma. In tissue taken from patients with SSc, the losses in the basement membrane integrity were found to be more than in the localized group (Fig. 1B).

Using skin samples from systemic sclerosis patients and healthy controls along with cell culture models of epithelial-mesenchymal transition (EMT), researchers found that TGF-ß signaling was active in keratinocytes (epidermal cells) from systemic sclerosis patients. Also, the loss of E-cadherin can induce intense EMT in keratinocytes in vitro via its effect on TGF-beta [26]. In their examination of skin fibrosis in morphea patients, Takahashi et al. [31] investigated the engagement of EMT. Their findings revealed a decrease in E-cadherin expressions in morphea, especially within the eccrine glands, as compared to the expressions observed in healthy skin. Meanwhile, there was an elevation in the expressions of TGF-ß1, Snail1, and fibronectin in morphea cases.

In this investigation, our objective was to elucidate the cutaneous expression patterns of E-Cadherin, CTGF, and TGF-ß in SSc and localized scleroderma, aiming to highlight distinctions in the pathogenesis of these two conditions (Fig. 1C–E). Additionally, we explored variations in both TGF-ß1 levels and mRNA expression between patients and healthy individuals. Our findings revealed a reduction in E-cadherin expression in the basal layer of the skin in both systemic and localized scleroderma groups when compared to the control group (Fig. 1D). Furthermore, no discernible involvement was noted in the stratum spinosum among the systemic sclerosis patients. Conversely, TGF-ß levels were observed to be lower in the stratum spinosum of systemic sclerosis patients compared to both the control group and those with localized scleroderma (2+ versus 1+) (Fig. 1E).

Systemic sclerosis is characterized by alterations in the vasculature, activation of the immune system, and the development of tissue fibrosis. Previous research has implicated the activation of the interferon system in the pathogenesis of this condition. Connective tissue growth factor (CTGF/CCN2) is a functional pro-

tein that plays a crucial role in regulating MMPs and TIMPs, as well as in the synthesis and degradation of the extracellular matrix. The significance of CTGF overexpression in contributing to vascular changes and fibrosis in systemic sclerosis has been substantiated [32]. Studies have demonstrated that CTGF serves as both a mediator and a marker in fibrotic processes. Liu et al. [30] highlighted the overexpression of CTGF (CCN2) specifically in the endothelial cells of individuals diagnosed with systemic sclerosis.

In this study, CTGF had less involvement (1+) in the stratum basale in the control group; more involvement (2+) in the localized scleroderma in patient group, as well as involvement in the dermis (+); and involvement was found (2+) in the stratum basale and spinosum in the systemic group. There was no involvement in the dermis in the systemic group (Fig. 1C).

Systemic sclerosis is a challenging-to-treat condition associated with significant morbidity and mortality. The pathogenesis of this disease is believed to involve endothelial cell activation and apoptosis, although the precise mechanisms are not fully understood. Studies have demonstrated that serum samples from individuals with systemic sclerosis can induce endothelial activation and apoptosis in cultures involving endothelial cells and neutrophils. Notably, this effect is primarily dependent on interleukin-6 (IL-6) [33, 34].

In our study, TUNEL staining performed to demonstrate cell apoptosis showed 3+ involvement in the stratum basale and stratum spinosum in the localized group. In SSc, involvement of the stratum basale and stratum spinosum was detected as 3+. These findings were not observed in healthy tissue samples (Fig. 1F).

Fibrosis, a condition characterized by pathological scarring, exerts its detrimental impact on the skin, liver, kidneys, and lungs, leading to severe morbidity. Currently, no cure exists for fibrosis, prompting recent molecular studies to unravel its underlying pathogenesis. Central to fibrogenesis is TGF-ß, with signaling mechanisms instigating the fibrogenic response of TGF-ß. [21]. TGF-ß is effective in the increase in collagen production and the transformation of fibroblast into myofibroblast. At the same time, TGF-ß regulates genes that play a key role in pathological fibrosis [35]. Additionally, pro-fibrotic proteins such as ET-1 and CTGF contribute to enhancing TGF-ß signaling. In systemic SSc, fibrosis arises from inflammation and vasculopathy, resulting in pathological scar formation [36].

In the study results, the levels of TGF-ß1 in patients were notably elevated compared to the control group (926±603.2 vs. 370±270, p=0.017). Examination of tissue analyses revealed an increased TGF-ß staining in the systemic group compared to both the localized and healthy tissues (Fig. 1E). TGF-ß1 expression was significantly higher in the systemic group in comparison to both localized and healthy tissues (Table 1). Based on these findings, it was deduced that TGF-ß plays a pivotal role in the fibrosis observed in scleroderma disease, and the heightened expression is linked to its pathogenesis. Additionally, the study demonstrated that TGF-ß has the potential to limit the pathological response in skin fibroblasts from individuals with localized scleroderma.

Conclusion

In this research aimed at unraveling the disease's pathogenesis, distinct characteristics were revealed at the tissue level through histopathological examinations between the two clinically diverse subgroups. Given the ongoing controversy surrounding the treatment of this disease, we believe that the obtained results will contribute to uncovering novel possibilities for treatment.

Ethics Committee Approval: The Ege University Faculty of Medicine Clinical Research Ethics Committee granted approval for this study (date: 03.07.2012, number: B.30.2.EGE.0.20.05.00/OY/1131/498).

Authorship Contributions: Concept – BS, ST, BYA, HA; Design – BS, ST, BYA, HA; Supervision – BS, ST, BYA, HA; Fundings – ST, BYA; Materials – ST, BYA; Data collection and/or processing – ST, BYA; Analysis and/or interpretation – BS, HA; Literature review – BS; Writing – BS; Critical review – BS, ST, BYA, HA.

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