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Original Article

Increased expression of guanylate binding protein-1 in lesional skin of patients with cutaneous lupus erythematosus

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Abstract: The large GTPase human guanylate binding protein-1 (GBP-1) is a key mediator of angiostatic effects of inflammation and is induced by interferon (IFN)-α and IFN-γ in endothelial cells (ECs). The aim of this study was to investigate whether GBP-1 is a marker of skin lesions in patients with cutaneous lupus erythematosus (CLE). Western blotting revealed that GBP-1 was *in vitro* induced by IFN-α and -γ in primary keratinocytes obtained from healthy controls. Moreover, we found that this protein was expressed by keratinocytes and ECs in primary and ultraviolet (UV)-induced skin lesions from patients with various subtypes of CLE, when compared to non-lesional skin. No GBP-1 expression was noted in skin biopsy specimens 24 or 72 h after UV irradiation prior to lesion formation in patients with CLE or in healthy control specimens with or without UV irradiation. Initial findings suggest that GBP-1 is not expressed in other skin diseases with different inflammatory aetiology, such as atopic dermatitis. We conclude that GBP-1 expression is closely associated with skin lesions in patients with CLE, suggesting a contribution of GBP-1 in the pathogenesis of this disease.

Key words: guanylate binding protein – inflammation – interferon – lupus erythematosus – skin – UV light

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Introduction

Lupus erythematosus (LE) is a chronic inflammatory autoimmune disease with heterogeneous clinical presentation. In contrast to the multi-organ disease of systemic LE (SLE), the manifestations of cutaneous LE (CLE) are primarily confined to the skin. Clinical manifestation of CLE varies widely, making it necessary to classify different subtypes of the disease, such as acute CLE (ACLE), subacute CLE (SCLE), chronic CLE (CCLE), and intermittent CLE (ICLE) (1). CCLE is further subdivided into discoid lupus erythematosus (DLE), lupus erythematosus panniculitis (LEP), and chilblain lupus erythematosus (CHLE). The complexity of these subtypes hampers understanding of the underlying pathological mechanisms, in addition to implying a multifactorial pathogenic course.

Recent studies have further demonstrated that type I interferons (IFNs), such as IFN-α and -β, play an important role in the pathogenesis of CLE (2). Interestingly, strong expression of myxovirus protein A (MxA), a protein specifically induced by type I IFNs, has been observed in lesional skin of patients with CLE, along with large numbers of infiltrating $CXCR3^+$ lymphocytes (3). Natural IFN-α-producing cells, also termed plasmacytoid dendritic cells (pDCs), have been detected in CLE lesions (4,5) and are associated with the presence of MxA (3). These findings suggest that local IFN-α production by pDCs promotes T-helper 1 (Th1)-biased inflammation. In addition, Wenzel et al. (6) showed that the expression pattern of IFN-inducible proteins reflects the histological distribution of infiltrating immune cells in different subtypes of CLE. These results demonstrate a close morphological association between IFN-inducible protein expression and the distribution of $CXCR3^+$ lymphocytes, supporting the importance of IFN-driven inflammation in CLE (7).

Among the proteins most abundantly induced by IFNs in eukaryotic cells is guanylate binding protein-1 (GBP-1) (8). GBP-1 belongs to the large GTPase family, which consists of seven homologous members (9–11). GBP-1 is expressed by endothelial cells (ECs) exposed to IFN-α and -γ, interleukin (IL)-1α and -1β, and TNF-α both *in vitro* and *in vivo* (12,13) and mediates these proteins' potent anti-angiogenic effects (14–16). GBP-1 has been identified as a marker of the proinflammatory microenvironment dominated by these cytokines during such inflammation-associated skin diseases as psoriasis and Kaposi's sarcoma, as well as during adverse drug reactions (13). GBP-1 also inhibits EC proliferation and invasiveness (14,15) as well as the ability of ECs to spread and migrate (16), thus mediating the anti-angiogenic effects of inflammatory cytokines. Interestingly, the protein is secreted from cytokine-activated ECs, suggesting in addition an extracellular function which has to be determined (17). Recently, GBP-1 was found to be expressed in 32% of patients suffering from colorectal carcinoma and was correlated with improved cancer-related survival (18). In these patients, GBP-1 expression was associated with an IFN-dependent, Th1-like anti-angiogenic microenvironment.

To investigate the role of GBP-1 in CLE, we analysed its expression in tissues of patients suffering from various disease subtypes. Lesional and non-lesional skin biopsies from the same patients with CLE were studied, as well as skin biopsies from patients undergoing ultraviolet light (UV) light exposure. Additionally, the expression levels were compared with those of healthy controls and patients suffering from other skin diseases. Moreover, the

expression of other IFN-dependent proteins, such as MxA, CXCL9, and CXCL10, was analysed to identify potential coregulation with GBP-1.

Methods

Patients and skin biopsies

The diagnosis and classification of CLE were based on clinical and histopathological criteria as well as on serologic abnormalities according to the Duesseldorf Classification 2004 (1). In total, thirty-eight patients with CLE were included in the study: SCLE, five men and four women, 48.4 ± 19.5 years; DLE, nine men and five women, 48.4 ± 13.5 years; and lupus erythematosus tumidus (LET), seven men and eight women, 48.1 ± 17.0 years. None of these patients fulfilled four or more criteria of the American College of Rheumatology (ACR) for the classification of SLE at the time of analysis (19). Skin biopsy specimens were collected from primary skin lesions (e.g. face, chest, arms, back abdomen) of 31 patients with different stages of the disease (e.g. early acute, fully developed, and late chronic lesions), from fully developed skin lesions induced by UVB irradiation (1.5 minimal erythema dose [MED]) of five patients, and from non-lesional skin of nine patients with CLE. We also analysed skin biopsy specimens from two patients with CLE 24 and 72 h after a single dose of a combined UVA (60–100 J/cm^2) and UVB (1.5 MED) irradiation. For control purposes, skin biopsies from one untreated healthy control were examined 24 and 72 h after a single dose of UVA (60–100 J/cm^2) and UVB (1.5 MED) irradiation (Data S1). As a control, we also investigated skin biopsy specimens from five patients with atopic dermatitis (AD, three men and two women, 55.4 ± 26.5 years) as well as from 13 healthy controls. The ethics committee of the University of Heidelberg, Germany, approved the study, and it was conducted according to the ethical guidelines of our institution and the Helsinki Declaration.

Immunohistochemistry and evaluation

Staining of paraformaldehyde-fixed, paraffin-embedded tissue sections for GBP-1 was performed as previously described (13,14,18). In addition, MxA was used as a marker for type I IFN signalling (M143, 1:100; Prof. Otto Haller, University of Freiburg, Freiburg, Germany). Additionally, we analysed the lesional expression of the IFN-inducible chemokines CXCL9/Mig (MAB392; R&D Systems, Minneapolis, MN, USA) and CXCL10/IP10 (Clone 33036; R&D Systems), as well as their common receptor, CXCR3 (1C6, 1:100; PharMingen, San Diego, CA, USA). The expression of MxA, CXCL9, and CXCL10 was scored, respectively (0 = no expression; + = weak expression; ++ = moderate expression; +++ = strong expression), as previously described (3). Lymphocyte populations were counted per three high-power fields (magnification ×200) and the mean population was calculated.

Further details from the methodology such as provocative phototesting, isolation of primary keratinocytes, and stimulation with cytokines as well as Western Blotting are included in the supplementary material (Data S1).

Table 1. GBP-1 is upregulated in all CLE subtypes and is absent in atopic dermatitis and healthy controls

Disease	Skin biopsies (*n*)	GBP-1 expression (*n*)	Positive cell types[1]
Subacute cutaneous lupus erythematosus (SCLE)	6	6	EP, EC, IF
Discoid lupus erythematosus (DLE)	11	10	EP, EC, IF
Lupus erythematosus tumidus (LET)	5	5	EP, EC, IF
Atopic dermatitis	5	0	–
Healthy controls	13	0	–

[1]epithelial cell (EP), endothelial cell (EC), infiltrate (IF)

Results

GBP-1 expression is upregulated in all CLE subtypes but not in atopic dermatitis or healthy controls

Guanylate binding protein-1 expression in punch biopsies from skin of patients with SCLE (n = 6), DLE (n = 11), LET (n = 5), atopic dermatitis (n = 5), and healthy controls (n = 13) was investigated using a well characterized rat monoclonal antibody against human GBP-1 (13) (Fig. 1; Table 1). GBP-1 expression was increased in more than 95% of all patients with CLE (Table 1) and was associated with epithelial (Fig. 1a, arrowheads), endothelial (Fig. 1a, arrows), and infiltrating (Fig. 1a, open arrows; Table 1) cells. There was no difference in the expression of GBP-1 with regard to the stage of the disease (e.g. early acute, fully developed, and late chronic lesions) and the site of localization (e.g. face, chest, arms, back abdomen). In contrast, however, GBP-1 expression was absent from tissues of patients with atopic dermatitis or healthy controls (Fig. 1b; Table 1). In contrast, staining controls (isotype control or without primary antibody) were performed and yielded negative results (data not shown).

GBP-1 is expressed in lesional but not in non-lesional skin of patients with CLE and can be induced by IFNs in primary keratinocytes

Three pairs of lesional and non-lesional skin from patients of each CLE subtype (SCLE, DLE, and LET) were analysed for GBP-1 expression. GBP-1 was found to be expressed in all cases of lesion-

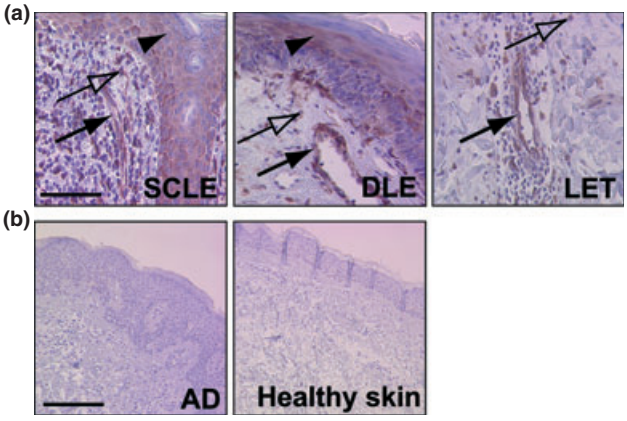

Figure 1. Guanylate binding protein (GBP)-1 expression is upregulated in all CLE subtypes but not in atopic dermatitis or healthy skin. Tissue sections from patients with (a) subacute cutaneous lupus erythematosus (SCLE, n = 6), discoid lupus erythematosus (DLE, n = 11), lupus erythematosus tumidus (LET, n = 5), and (b) atopic dermatitis (n = 5), as well as healthy controls (n = 13) were stained using a specific rat anti-human GBP-1 monoclonal antibody (clone 1B1). Brown colour indicates GBP-1 expression (arrows), while nuclei were counterstained with haematoxylin and are displayed in blue. Epithelial cells are indicated by arrowheads, endothelial cells by solid arrows, and infiltrating cells by open arrows. Scale bars = (a) 100 μm, (b) 250 μm.

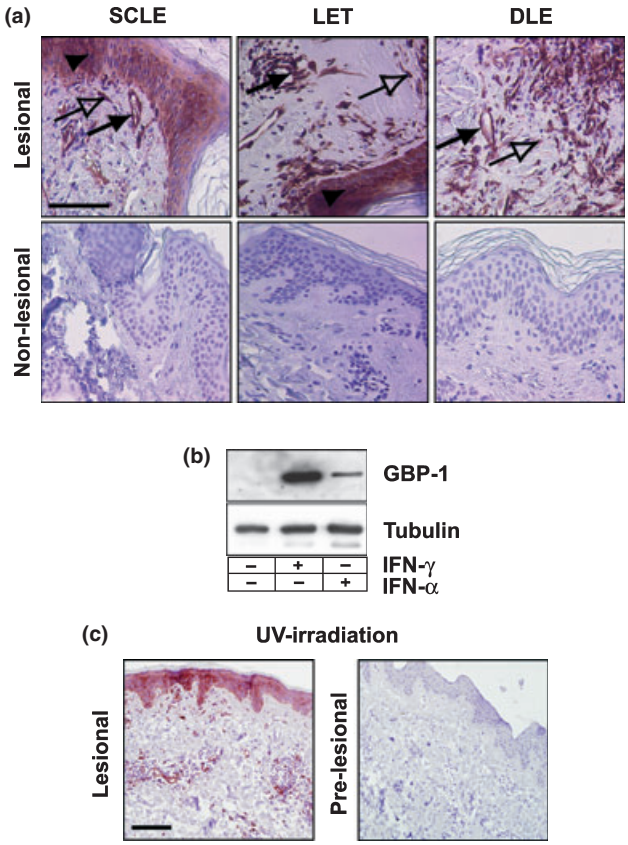

Figure 2. Guanylate binding protein (GBP)-1 is expressed in primary skin lesions and in fully developed UV-induced skin lesions of patients with CLE but not prior to lesion formation after UV irradiation. (a) Lesional and non-lesional skin of patients with subacute cutaneous lupus erythematosus (SCLE, $n = 3$), discoid lupus erythematosus (DLE, $n = 3$), and lupus erythematosus tumidus (LET, $n = 3$) was stained using a specific rat anti-human GBP-1 monoclonal antibody (1B1). Brown colour indicates GBP-1 expression (arrows), while nuclei were counterstained with haematoxylin and are displayed in blue. Epithelial cells are marked by arrowheads, endothelial cells by solid arrows, and infiltrating cells by open arrows. Scale bar = 100 μm. (b) Primary keratinocytes were isolated from healthy human donors and were either left untreated or stimulated for 24 h with 1000 U/ml IFN-α or 100 U/ml IFN-γ. Protein extracts were then isolated from these cells and Western blotting using a specific anti-human GBP-1 antibody (1B1) was performed. Tubulin was also blotted to demonstrate equal protein loading. (c) Specimens from the UV-induced, fully developed skin lesions ($n = 5$), and UV-irradiated skin prior to lesion formation ($n = 4$) of patients with CLE , as well as specimens from healthy controls that were irradiated with UV ($n = 2$, data not shown), were stained using a specific rat anti-human GBP-1 monoclonal antibody (1B1). Brown colour indicates GBP-1 expression (arrows), while nuclei were counterstained with haematoxylin and are displayed in blue. Scale bar = 250 μm.

al skin ($n = 9$; Fig. 2a; Table 2), while it was not expressed in non-lesional skin ($n = 9$) of the same patients (Fig. 2a, non-lesional; Table 2). The GBP-1-positive cell types in the lesional skin were epithelial (Fig. 2a, arrowheads), endothelial (Fig. 2a, arrows), and infiltrating (Fig. 2a, open arrows) cells, which were most likely monocytes/macrophages (Table 2).

To analyse whether IFN-α and -γ are inducers of GBP-1, primary keratinocytes from healthy controls were stimulated for 24 h with 1000 U/ml IFN-α or 100 U/ml IFN-γ. These IFN concentrations were previously described as the maximal doses required to induce GBP-1 expression (13). In Western blotting, GBP-1 was found to be induced by both cytokines (Fig. 2b). Notably, GBP-1 induction by IFN-γ was more pronounced than that by IFN-α.

Table 2. Guanylate binding protein (GBP)-1 expression is associated with lesional skin in patients with CLE

Disease subtype	GBP-1 expression		
	Lesional ($n = 3$)	Non-lesional ($n = 3$)	Positive cell types[1]
Subacute cutaneous lupus erythematosus (SCLE)	3	0	EP, EC, IF
Discoid lupus erythematosus (DLE)	3	0	EP, EC, IF
Lupus erythematosus tumidus (LET)	3	0	EP, EC, IF

[1]epithelial cell (EP), endothelial cell (EC), infiltrate (IF)

GBP-1 expression is induced in fully developed UV-induced lesions of patients with CLE but not prior to lesion formation

A photoprovocation test was performed to analyse whether GBP-1 expression is induced by UV exposure. Five patients with CLE were irradiated by UVB light according to a standardized protocol, and punch biopsies of skin were collected before and after formation of characteristic CLE lesions. GBP-1 expression was detected only in fully developed skin lesions of these patients, while expression was absent in skin biopsies both 24 and 72 h after UV irradiation prior to lesion formation (Fig. 2c; Table 3). Moreover, the protein was not expressed in healthy control specimens 24 or 72 h after UV irradiation (data not shown). This finding suggests that GBP-1 is only expressed in the fully developed lesions of patients with CLE and is not induced by UV irradiation in healthy controls or prior to lesion formation in patients with CLE.

GBP-1 expression is coregulated with other IFN-dependent proteins, such as MxA, CXCL9, and CXCL10

Finally, we investigated whether GBP-1 expression correlates with the increased expression of other IFN-dependent genes in patients with CLE. Five consecutive skin sections obtained from patients with CLE ($n = 6$) were stained for GBP-1, MxA, CXCL9, and CXCL10 expression. In addition, the expression of the CXCL9 and CXCL10 chemokine receptor, CXCR3, was analysed. GBP-1 was found to be expressed in the same tissue areas as MxA, CXCL9, and CXCL10 (Fig. 3). However, the cell types expressing GBP-1 partly differed from those expressing the other IFN-regulated genes. For example, GBP-1 is detected in

Table 3. Guanylate binding protein (GBP)-1 expression is induced in fully developed lesions of patients with CLE after UV irradiation

Disease subtype	Skin biopsies (n)	GBP-1 expression after UV irradiation (n)	Positive cell types[1]
UV-induced CLE skin lesions	5	4	EP, EC, IF
Prelesional UV-irradiated skin of patients with CLE	4	0	–
UV-irradiated skin of healthy controls	2	0	–

[1]epithelial cell (EP), endothelial cell (EC), infiltrate (IF)

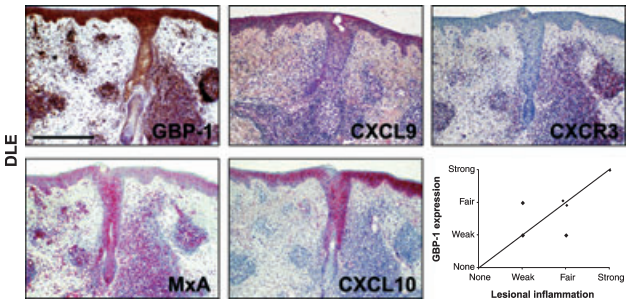

Figure 3. Guanylate binding protein (GBP)-1 expression is coregulated with other IFN-dependent proteins, such as MxA, CXCL9, and CXCL10, in patients with CLE. Lesional skin biopsy specimens from patients with CLE (n = 6) were stained for GBP-1, MxA, CXCL9, CXCL10, and CXCR3 using specific antibodies. Brown colour indicates GBP-1 expression while MxA, CXCL9, CXCL10, and CXCR3 expression is indicated by pink colour. The nuclei were counterstained using haematoxylin and are displayed in blue. Scale bar = 250 μm. GBP-1 expression and the inflammatory infiltrate was scored semi-quantitatively (no expression > weak expression > fair expression > strong expression).

epithelial cells, endothelial cells, and infiltrate, whereas CXCL9 is expressed by epithelial cells only. The strongest coregulation of expression was detected for GBP-1 and MxA, both expressed in epithelial cells, endothelial cells, and infiltrate. CXCR3 was also present in the same regions of the tissue. In addition, the level of GBP-1 expression was correlated with the degree of lesional inflammation.

Discussion

CLE is a chronic autoimmune disease characterized by skin inflammation. Recent studies have demonstrated that type I IFNs, such as IFN-α, play a crucial role in the pathogenesis of the disease. Here, we investigated the expression and function of GBP-1, one of the major IFN-inducible proteins in eukaryotic cells. The expression of this IFN-dependent large GTPase was analysed in skin biopsy specimens obtained from patients with different subtypes of CLE. Specimens collected after UV exposure, as well as biopsies from healthy controls, were also analysed. We observed that (i) GBP-1 was upregulated in all investigated CLE subtypes and (ii) its expression was solely detectable in the lesional skin of these patients. Moreover, (iii) GBP-1 was also present in fully developed UV-induced lesions but was not expressed after UV exposure or prior to lesion formation. In addition, (iv) the protein was not detected in skin biopsies obtained from healthy controls with or without UV irradiation or from patients with other skin diseases with different inflammatory aetiology, such as atopic dermatitis.

Moreover, the presence of type I IFNs, such as IFN-α, in skin lesions of patients with CLE has been well described and is known to encourage progression of this autoimmune disease (3,20–23). In contrast to CLE, acute lesions of atopic dermatitis of adult patients are characterized by elevated levels of Th2 cytokines and low levels of TNF-α, IL-1β, and IFN-γ (24,25). Moreover, GBP-1 expression is selectively upregulated by IFN-α and -γ, TNF-α, and/or IL-1α and -1β and is not induced by any other chemokine (MCP-1, MIP-1β, PF-4, CXCL10/IP-10, SDF-1α), angiogenic growth factor (VEGF, bFGF, angiopoietin-2, PDGF-B/B), or cytokine (IL-4, -6, -10, -18, oncostatin M) tested so far (13). Therefore, our data are consistent with the fact that GBP-1 expression is absent in the skin of atopic dermatitis and healthy patients. The increased expression of GBP-1 in skin lesions of patients with CLE indicates that a specific inflammatory microenvironment may be present in the disease, characterized by increased levels of IFNs, TNF-α, and/or IL-1. These proinflammatory cytokines are known to activate ECs, suggesting that the vascular system is involved in CLE. However, it is still to be determined whether GBP-1 is not only a marker of activation of these cells but also plays a regulatory role in CLE.

Interestingly, GBP-1-positive cell types in CLE included ECs, infiltrating cells, and skin epithelial cells, such as keratinocytes. A high association of GBP-1 with ECs and infiltrating cells *in vivo* has been shown previously (13,14), but this is the first report of association with skin epithelial cells. It has been described that pDCs are the major cellular source of IFNs in CLE and accumulate in high amounts in the skin of patients with the disease (4). Therefore, the epithelial cells in the skin of patients with CLE are likely constantly exposed to this highly IFN-dominated microenvironment, which in turn may trigger the upregulation of GBP-1 in these cells.

Moreover, it has been demonstrated that UVB irradiation of primary human keratinocytes in the presence of proinflammatory cytokines, such as IL-1 (26), TNF-α (27), and IFN-γ, significantly enhances the expression of the chemokines CCL5, CCL20, CCL22, and CXCL8 (28). In addition, the CXCR3 ligands CXCL9, CXCL10, and CXCL11 have been identified as the most abundantly expressed genes in patients with CLE (2,6). In the present study, GBP-1 was found to be coexpressed with other IFN-dependent genes, such as MxA and the antiangiogenic chemokines CXCL9 and CXCL10. Notably, MxA expression is mainly IFN-α-dependent (29), whereas CXCL9 and CXCL10 can be induced by either IFN-α or -γ, similar to GBP-1 (30,31). In addition, the CXCL9 and CXCL10 chemokine receptor CXCR3 (32) was coexpressed in areas of GBP-1/CXCL9/CXCL10 expression. CXCL9 and CXCL10 are not only the most abundantly expressed genes in patients with CLE but are also well characterized as anti-angiogenic chemokines in diseases such as metastatic renal cell carcinoma (33,34). In colorectal carcinoma, the coregulation of GBP-1 with CXCL9 and CXCL10 has been described (18). Interestingly, GBP-1-positive patients were characterized by an increased cancer-related survival of 16.2% and a reduced relative risk of cancer-related death by 50% when compared to GBP-1-negative patients. It was further demonstrated that in colorectal carcinoma with high GBP-1 expression, angiogenesis was reduced (18). This suggests that during high GBP-1/CXCL9/CXCL10 expression, angiogenesis might be reduced in the skin of patients with CLE as GBP-1 was also found to be associated with endothelial cells in the disease. In addition, the reduced angiogenesis may be at least partially caused by reduced neovascularization. The functional role of GBP-1 expression in epithelial cells remains to be determined.

The exclusive expression of GBP-1 in the genuine and fully developed UV-induced skin lesions of patients with CLE suggests that GBP-1 may play an important role in disease pathogenesis. It has to be determined whether GBP-1 can be used as a diagnostic marker or as a promising target for therapeutic intervention.

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Authors' Contributions

AK, EN, MH and MS designed research; AK, CK, EN and JW performed experiments; AK, EN, JW, MH and MS analysed data; AK, EN and JW prepared the manuscript. The final manuscript was approved by all authors.

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Supporting Information

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Data S1. Supplemental material.

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