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A functional interleukin-1β gene polymorphism is associated with chronic periodontitis in a sample of Brazilian individuals

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Background: Interleukin-1 beta (IL-1β) is a potent inflammatory mediator and an important polymorphism in the *locus* +3954 (C/T) of the human *IL1B* gene has been shown to affect the levels of this cytokine. This functional polymorphism has been associated with the establishment of inflammatory diseases, including periodontal disease, in European, Asian and North American populations.

Objective: The aim of this study was to investigate the association between the *IL1B* (+3954) gene polymorphism and the occurrence of different clinical forms of periodontitis in a sample of Brazilian individuals.

Methods: This study employed a cross-sectional design involving individuals from the State of Minas Gerais in the south-eastern region of Brazil. Genomic DNA was obtained from oral swabs of 129 individuals and amplified using the polymerase chain reaction (PCR) with specific primers flanking the *locus* +3954 of *IL1B*. PCR products were submitted to restriction endonuclease digestion and analyzed by polyacrylamide gel electrophoresis, to distinguish alleles T and C of the *IL1B* gene, allowing for the determination of the genotypes and detection of the polymorphism.

Results and Conclusions: The chronic periodontitis group displayed a higher percentage of the T allele (28%) when compared to the aggressive periodontitis group (10.7%, $\chi^2 = 5.24$, $p = 0.02$, OR = 0.31, CI = 0.11–0.88) and to control group (8.7%, $\chi^2 = 7.11$, $p = 0.007$, OR = 0.24, CI = 0.08–0.73). Our data suggested that the polymorphism in the *locus* +3954 of *IL1B* gene could be a risk factor for chronic periodontitis in a sample of Brazilian individuals.

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Periodontal disease is an inflammatory illness that represents the main cause of tooth loss in developed countries, with increasing prevalence in the developing world (1). Although the presence of gram-negative bacteria is essential for initiating and perpetuating periodontal disease, environmental as well as genetic factors contribute to individual variations in the etiology and course of disease (2). Studies in twins have indicated that a substantial portion of this inter-individual variability of periodontal parameters may be attributable to genetic factors (3, 4). Several reports indicate that polymorphisms in the interleukin-1 beta (IL-1β) gene cluster may influence this variation and, thus, the severity of periodontal disease (5–7).

IL-1 is a pro-inflammatory cytokine that plays a pivotal role in several

chronic diseases. This cytokine is a primary activator of early chemotactic cytokines, as well as of the expression of adhesion molecules that facilitate migration of leucocytes into tissues. IL-1 is also known to be one of the most active stimulators of osteoclastic bone reabsorption (8).

There are three genes that regulate the production of IL-1: *IL1A*, *IL1B* and *IL1RN* (9). These genes are located on chromosome 2q13. Genes *IL1A* and *IL1B* control the production of the pro-inflammatory proteins, IL-1α and IL-1β, respectively. *IL1RN* controls the synthesis of an antagonist protein (IL-ra) (10). Polymorphism in the +3954 *locus* (due to nomenclature change, the polymorphism at *IL1B* +3953 is now referred to as *IL1B* +3954 10), of the *IL1B* gene has been associated with an increased production of this cytokine. Homozygous individuals for the T allele produce a four-fold higher amount of IL-1β compared to individuals displaying the CC genotype (11). It has recently been suggested that this polymorphism may explain why some people have a more vigorous response than others to the same stimulus (8).

Several studies have evaluated gene polymorphisms in individuals with periodontitis in distinct populations. Kornman *et al.* (5) demonstrated that the occurrence of *IL1A* (–889) and *IL1B* (+3954) polymorphisms simultaneously was associated with a severity of chronic periodontitis in non-smoker Caucasians. Walker *et al.* (12) observed a high prevalence of *IL1B* (+3954) allele C in the African-American population and concluded that this polymorphism would provide little diagnostic or predictive information for localized aggressive periodontitis. As the frequency of many genetic alleles varies between ethnic groups and because contradictory results may be observed (10), the aim of this study was to describe the prevalence of the *IL1B* (+3954) gene polymorphism in a sample of Brazilian patients with different clinical forms of periodontitis and evaluate the association of this polymorphism with the severity of disease.

Material and methods

Patients

The study employed a cross-sectional design involving individuals from the State of Minas Gerais in the southeastern region of Brazil. A total of 129 patients receiving treatment at the Dentistry School, Federal University of Minas Gerais, were included in this study. The patients were stratified into three groups: subjects with aggressive periodontitis ($n = 46$), subjects with chronic periodontitis ($n = 52$) and healthy volunteers as the control group ($n = 31$). Patients in the aggressive periodontitis group were 15–46 years old and exhibited highly destructive forms of periodontitis with evidence of early onset. Patients in the chronic periodontitis group were 27–67 years old and exhibited inflammation, loss of clinical attachment due to destruction of periodontal ligament and loss of the adjacent supporting bone. All patients in the chronic periodontitis group had at least three teeth exhibiting sites of clinical attachment loss in at least two different quadrants. Diagnosis of disease was made considering the patient's medical and dental histories, radiographic findings and observations of clinical signs and parameters, including probing depth, assessment of clinical attachment loss, observation of tooth mobility, bleeding on probing and presence of plaque/calculus. Measurements of probing depth and attachment level were assessed at six locations around each tooth. The severity of disease was characterized on the basis of the amount of clinical attachment loss, within each clinical form. Patients exhibiting clinical attachment loss ≥ 5 mm were considered with severe and those exhibiting clinical attachment loss ≥ 3 mm to < 5 mm were considered with moderate periodontitis. Clinical diagnosis and determination of disease severity were based on criteria established in 1999 at the International Workshop for a Classification of Periodontal Diseases and Conditions (13). Control healthy individuals included in the study were 21–70 years old and did not have disease at the time of sample collection, and also did not present a history of previous periodontal disease; as determined by absence of clinical attachment loss and no sites with probing depth > 3 mm.

A questionnaire was applied to all individuals enrolled in this study, in order to obtain information regarding dental history, family history of periodontal disease, smoking habit, as well as general health concerns. Use of orthodontic appliances, chronic usage of anti-inflammatory drugs, history of diabetes, hepatitis or HIV infection, immunosupressive chemotherapy, bleeding disorders, severely compromised immune function, pregnancy or lactation were regarded as exclusion criteria. Except for the presence of periodontitis, the patients included in this study were systemically healthy. Because tobacco smoking is an important risk factor for periodontitis, we also analyzed our data taking the habit of smoking under consideration. 'Smokers' were defined as current smokers/former smokers (more than 10 cigarettes/day) and 'non-smokers' included individuals that had never smoked. Table 1 summarizes the

Table 1. Characteristics of the study groups

Clinical forms	Aggressive periodontitis	Chronic periodontitis	Healthy control
Number of individuals	46	52	31
Gender			
Male (%)	16 (34.8)	19 (36.5)	10 (32.3)
Female (%)	30 (65.2)	33 (63.5)	21 (67.7)
Clinical attachment loss mean ± SD (mm)	6.27 ± 1.68	5.68 ± 1.26	–
Smoking status			
Non-smokers (%)	28 (60)	25 (48.1)	29 (93.5)
Former smokers (%)	9 (20)	15 (28.8)	2 (6.5)
Smokers (%)	9 (20)	12 (23.1)	–

patient data, as well as their classification into different groups.

This study was approved by the University's Ethics Committee (n°003/03) and a signed informed consent was obtained from all participants.

Sample collection and DNA extraction

Epithelial cells were obtained through a oral swab performed with a sterile plastic spatula. After gentle scraping of oral mucosa, the tip of the spatula was immediately immersed in 2-ml sterile microtubes containing 1500 µl of Krebs buffer (NaCl 20%, KCl 2%, $CaCl_2$ 2%, H_2O 2%, $MgSO_4$, KH_2PO_4, $C_6H_{12}O_6$). DNA extraction was performed as described by Boom *et al.* (14) and modified as follows. A pellet of epithelial cells was obtained by centrifugation at 200 ***g*** for 5 min. The supernatant was removed and 20 µl of silica (SiO_2, Sigma, St Louis, MO, USA) and 450 µl of lysis buffer (6.0 M GuSCN, 65 mM Tris-HCl pH 6.4, 25 mM EDTA and 1.5% Triton X-100) were added to the microtubes. Samples were homogeneized by using a Vortex and incubated for 30 min at 56°C. After this incubation, samples were submitted to another centrifugation and the supernatant was discharged. The pellet obtained (with DNA adsorbed on the silica) was washed twice with 450 µl of washing buffer (6.0 M GuSCN, 65 mM Tris-HCl pH 6.4), twice with 450 µl of 70% ethanol, once with 450 µl acetone and dried at 56°C for 20 min. Finally, 100 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) was added and incubated at 56°C for 12 h to release the DNA. After incubation, the solution was homogenized, centrifuged and the supernatant containing DNA transferred to a new tube.

Polymerase chain reaction and restriction endonuclease digestion

IL1B (+3954) polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers were 5′-CTCAGGTGTCCTCGAAGAAATCAAA-3′ and 5′-GCTTTTTTGCTGTGAGTCCCG-3′ with expected PCR product size of 194 bp, as described previously (5). PCR was carried out in a total volume of 50 µl, containing 10 µl of solution DNA, Pre-mix buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1% Triton X-100, 1.5 mM $MgCl_2$, deoxynucleoside triphosphates, *Taq* DNA polymerase) and primers (20 pmol/reaction). The amplification conditions consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 35 s and 72°C for 30 s. The run was terminated by final elongation at 72°C for 5 min. Amplification was performed in a PTC-100-60 thermocycler (MJ Research, Waltman, MA, USA). The products were digested with 5 U of *TaqI* at 65°C for 4 h and obtained 97 + 85 + 12 bp DNA products for allele C and 182 + 12 bp DNA products for allele T. The visualization was performed in a 10% polyacrylamide gel eletrophoresis.

Statistical analysis

Statistical analysis of data was performed using the JMP statistical software (SAS, Cary, NC, USA). The chi-squared test was used to compare the genotypes distributions between control and chronic periodontitis groups, between control and aggressive periodontitis groups, and between chronic periodontitis and aggressive periodontitis groups [3×2 contingency table, degrees of freedom (d.f.) = 2). The C/T allele and T+/T– genotype distribution between control and chronic periodontitis groups, between control and aggressive periodontitis groups, and between chronic periodontitis and aggressive periodontitis groups was assessed in a 2×2 contingency table (d.f. = 1). Odds ratio (OR) with 95% confidence intervals (CI) in 2×2 comparisons were calculated to determine the strength of the association. Multivariate logistic regression was utilized to assess the relationship of genotype to disease status while adjusting for potencial confounders, such as smoking history. To exclude the possible confounding effect of smoking, in a second analysis we excluded smokers from all the different clinical groups. A p-value < 0.05 was considered significant.

Results

The genotype and allele distributions of the *IL1B* (+3954) polymorphism are shown in Tables 2 and 3, respectively. The evaluation of the genotype distribution was performed comparing periodontal patients with different clinical forms and individuals without disease, considering or not the habit of smoking. There was a significant difference in the genotype distribution when comparing the control group with the chronic periodontitis group ($\chi^2 = 8.13$, $p = 0.01$), as well as comparing aggressive periodontitis with chronic periodontitis group ($\chi^2 = 6.24$, $p = 0.04$), considering only non-smokers (Table 2). No statistical difference was observed when comparing control and aggressive periodontitis

Table 2. Distribution of the *IL1B* (+3954) genotypes in the study groups

Genotype	Healthy control	Aggressive periodontitis	Chronic periodontitis
Non-smokers			
CC (%)	24 (82.76)	22 (78.57)	12 (48.0)
CT (%)	5 (17.24)	6 (21.43)	12 (48.0)
TT (%)	–	–	1 (4.0)
Non-smokers + smokers			
CC (%)	24 (77.42)	34 (73.91)	29 (55.77)
CT (%)	7 (22.58)	12 (26.09)	20 (38.46)
TT (%)	–	–	3 (5.77)

Statistical difference (3×2 contingency table):
non-smokers (control vs. chronic periodontitis): $\chi^2 = 8.13$, $p = 0.01$;
non-smokers (aggressive periodontitis vs. chronic periodontitis): $\chi^2 = 6.24$, $p = 0.04$.
χ^2, chi-squared.

Table 3. Distribution of the *IL1B* (+3954) alleles in the study groups

Allele	Healthy control	Aggressive periodontitis	Chronic periodontitis
Non-smokers			
C (%)	53 (91.3)	50 (89.3)	36 (72)
T (%)	5 (8.7)	6 (10.7)	14 (28)
Non-smokers + smokers			
C (%)	55 (88.7)	80 (86.9)	78 (75)
T (%)	7 (11.3)	12 (13.1)	26 (25)

Statistical difference (2 × 2 contingency table):
non-smokers (control vs. chronic periodontitis): $\chi^2 = 7.11$, $p = 0.007$, OR = 0.24, CI = 0.08–0.73;
non-smokers (aggressive periodontitis vs. chronic periodontitis): $\chi^2 = 5.24$, $p = 0.02$, OR = 0.31, CI = 0.11–0.88;
non-smokers + smokers: $\chi^2 = 4.99$, $p = 0.02$, OR = 0.26, CI = 0.07–0.88.
χ^2, chi-squared; OR, odds ratio; CI, confidence interval.

groups. Results of multiple logistic regression analysis did not show a significant difference among the groups when the smokers were included (Table 2). The data indicate that the frequencies of CT and TT genotypes may be related to chronic periodontitis in non-smokers. With regards to allele distribution, statistical differences were observed when comparing control and chronic periodontitis groups ($\chi^2 = 7.11$, $p = 0.007$, OR = 0.24, CI = 0.08–0.73), as well as comparing aggressive periodontitis and chronic periodontitis groups ($\chi^2 = 5.24$, $p = 0.02$, OR = 0.31, CI = 0.11–0.88), considering only non-smokers (Table 3). The chronic periodontitis group displayed a higher percentage of the T allele (28%) when compared to aggressive periodontitis (10.7%) and control (8.7%) groups. When smokers were included, a statistical difference in allele distribution and a higher percentage of T allele frequency were still observed in the chronic periodontitis group when compared to control groups ($\chi^2 = 4.99$, $p = 0.02$, OR = 0.26, CI = 0.07–0.88) (Table 3). The analysis of the frequency of T+ individuals vs. T– individuals (in other words, the presence of T allele) between the groups was significantly different when analyzing non-smokers. These data are shown in Fig. 1 and demonstrate the association of the T+ genotype with the chronic periodontitis group in non-smokers.

When we evaluated the severity of periodontitis by stratifying the groups according to clinical attachment loss, we observed that non-smoker individuals with severe aggressive periodontitis displayed a suggestive bias ($\chi^2 = 3.25$, $p = 0.07$, OR = 6.33, CI = 0.84–47.31) for the frequency of T– genotypes as compared to individuals with moderate aggressive periodontitis (Table 4). No difference was observed when smokers were included. With regards to the chronic periodontitis group, no statistical difference was observed between the moderate and severe groups (Table 4). Finally, when considering only individuals with the severe clinical attachment loss from both clinical forms of periodontitis, we observed that non-smoker individuals with severe chronic periodontitis displayed a suggestive bias ($\chi^2 = 3.33$, $p = 0.06$, OR = 4.22, CI = 0.85–20.84) for the T+ genotype as compared to individuals with severe aggressive periodontitis (Table 4). No differences were observed when smokers were included (Table 4).

Discussion

The observation that genetic polymorphisms may be associated with the

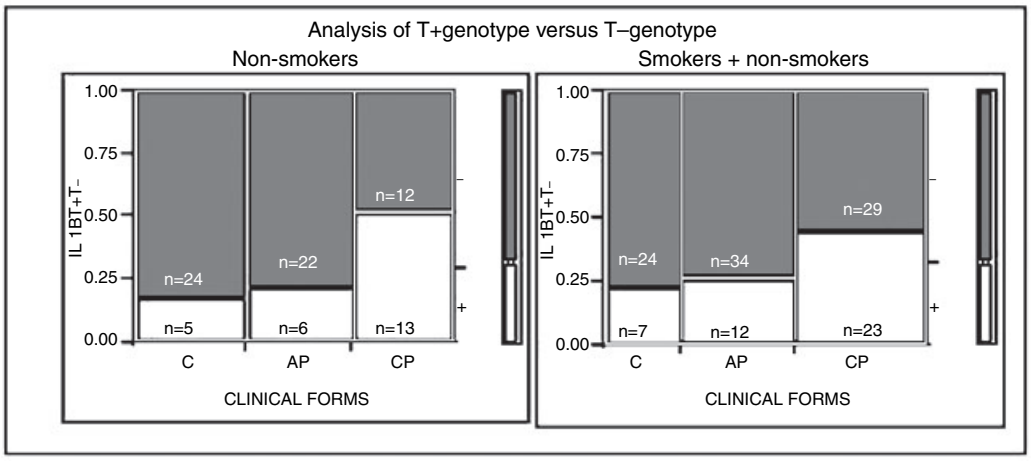

Fig. 1. Analysis of individuals with T+ genotype vs. individuals with T– genotype for the different clinical forms of periodontitis. White boxes are representative of the positive T genotype and black boxes are representative of the negative T genotype. Statistical difference (2 × 2 contingency table): non-smokers (control vs. chronic periodontitis): $\chi^2 = 7.46$, $p = 0.006$, OR = 5.2, CI = 1.50–18.01; non-smokers (aggressive periodontitis vs. chronic periodontitis): $\chi^2 = 5.45$, $p = 0.01$, OR = 0.25, CI = 0.76–0.83. C, control; AP, aggressive periodontitis; CP, chronic periodontitis; χ^2, chi-squared; OR, odds ratio; CI, confidence interval.

Table 4. Distribution of the *IL1B* (+3954) genotypes in the study groups, considering the severity of disease

Genotype	Non-smokers + smokers		Non-smokers	
	Aggressive periodontitis	Chronic periodontitis	Aggressive periodontitis	Chronic periodontitis
Severe				
CC (%)	29 (76.3)	24 (63.2)	19 (86.4)	9 (60)
CT (%)	9 (23.7)	12 (31.6)	3 (13.6)	6 (40)
TT (%)	–	2 (5.2)	–	–
Moderate				
CC (%)	5 (62.5)	5 (38.5)	3 (50)	3 (33.3)
CT (%)	3 (37.5)	7 (53.8)	3 (50)	5 (55.6)
TT (%)	–	1 (7.7)	–	1 (11.1)

Patients exhibiting clinical attachment loss ≥ 5 mm were considered with severe and those exhibiting clinical attachment loss ≥ 3 mm to < 5 mm were considered with moderate periodontitis. Statistical analysis (2 × 2 contingency table):
non-smokers (severe aggressive periodontitis vs. moderate aggressive periodontitis): $\chi^2 = 3.25$, $p = 0.07$, OR = 6.33, CI = 0.84–47.31;
non-smokers (severe aggressive periodontitis vs. severe chronic periodontitis: $\chi^2 = 3.33$, $p = 0.06$, OR = 4.22, CI = 0.85–20.84.
χ^2, chi-squared; OR, odds ratio; CI, confidence interval.

establishment of distinct immune responses to microbial infections has enhanced the interest in identifying gene polymorphisms associated with diseases (12). Many studies have reported associations between cytokine gene polymorphisms and periodontitis in distinct populations (5, 7, 15). The frequency of many genetic alleles varies between ethnic groups, and several studies have found contradictory results when comparing among distinct populations (10). Thus, the analysis of gene polymorphisms in a sample of the Brazilian individuals represents an important advance in the study of periodontal disease in Brazil. In this study, the organization of the sample of Brazilian individuals into ethnic groups was not performed due to the strong miscegenation among Brazilians. Recent articles have not recommended grouping Brazilians into ethnic groups based on color, race and geographical origin (16, 17). This study (17) has shown that Brazilian individuals classified as 'white' or 'black' have significantly overlapping genotypes, probably due to miscegenation. In our study, all the patients and controls were selected from the same geographic area, and were at the same socio-economic level. Moreover, the individuals analyzed in our study, from Minas Gerais State, are as representative of the Brazilian population as possible, as shown by Parra *et al*. (17).

In this study, we evaluated a polymorphism in the *locus* +3954 (C/T) of *IL1B* gene in a sample of the Brazilian population suffering or not from periodontal disease and found an association between the occurrence of the polymorphism and chronic periodontitis. This finding is in accordance with Rogers *et al*. (18), who observed that the *IL1B* (+3954) polymorphism alone was associated with chronic periodontitis in Caucasian patients resident in Western Australia. However, these authors did not report any relationship with the habit of smoking in the analyzed patient population. With regards to aggressive periodontitis and the gene polymorphism, no association was observed in our study. Our results are in accordance with Hodge *et al*. (19) and Tai *et al*. (20), who observed no significant differences in frequencies of the alleles between aggressive periodontitis patients and the control group in European Caucasian and in Japanese individuals, respectively. However, contradictory data were reported by Parkhill *et al*. (6), who observed an increased frequency of the C allele in aggressive periodontitis Caucasian patients when compared with the control group.

The *IL1B* (+3954) polymorphism is widely studied considering the severity of periodontitis. Kornman *et al*. (5) demonstrated an association of positive genotypes [occurrence of *IL1A* (–889) and *IL1B* (+3954) polymorphisms simultaneously] with severe chronic periodontitis in non-smoker Caucasian patients, and McDevitt *et al*. (2) reported that non-smokers or 'former light smokers', as defined by the authors, who displayed positive genotypes were more likely to have moderate–severe periodontitis. It has been mentioned that genetic polymorphisms likely influence susceptibility to periodontitis, and its clinical manifestations through the accumulated effect of multiple polymorphisms (10). However, single polymorphisms have been associated with severity of periodontitis, as reported by Gore *et al*. (7), who observed that frequencies of the T allele of the *IL1B* (+3954) polymorphism in Caucasians was significantly increased in severe periodontitis patients as compared to mild periodontitis patients, but not increased as compared to healthy individuals. Our results do not indicate an evident association of this polymorphism with severe chronic periodontitis, although a higher frequency of T+ genotype was detected when compared with severe aggressive periodontitis. With regards to aggressive periodontitis, the higher frequency of T– genotype in non-smoker individuals with the severe form of disease is consistent with the clinical feature of the lack of an overt inflammation observed in many cases (21).

It is a consensus in the literature concerning periodontitis that smoking is an important risk factor for the establishment of the disease. However, studies have shown that smokers with periodontitis have less clinical inflammation and gingival bleeding compared with non-smokers (22). This may be explained by the fact that smoke byproducts exert local vasoconstriction, reducing blood flow, edema and clinical signs of inflammation (22). In this study, we observed that the genetic association with the chronic periodontitis was more evident when smokers were excluded from the study

groups, confirming the importance of this risk factor and suggesting that its effect is strong even in subjects who are not genetically susceptible to disease. This data suggests that the smoking-related risk can often obscure the polymorphism-related risk, as described by Kornman *et al.* (5).

One plausible interpretation for the association of the *IL1B* gene polymorphism and periodontal disease is based on the fact that the evaluated polymorphism has been described as a functional polymorphism (11). Thus, the polymorphic genotypes would directly influence the disease pathogenesis via an effect on cytokine synthesis. An exacerbated expression of IL-1 could lead to higher inflammation and tissue destruction. However, results observed in various studies reflect differences in the role of *IL1B* polymorphism in pathogenesis of aggressive and chronic periodontitis, further suggesting that genetic differences may be related to different types of periodontal diseases (10, 12).

In our study, one possible limitation consists of the fact that some of the individuals who were classified, at the time of our study, as healthy might develop signs of periodontal disease in the future. However, based on the incidence of periodontal disease in Brazil (about 10%) (23), we would expect, at most, three individuals in our control group to develop chronic periodontitis. Thus, these individuals could bias our study. However, the loss of three individuals from our control group would not invalidate our findings. If anything, the loss of three individuals would strengthen the observed association. Finally, in many periodontal studies, age-matched studies between patients and control have not been considered necessary because the genetic patterns do not change with age (24, 25).

In conclusion, the present study shows that the polymorphism in the *locus* +3954 (C/T) of *IL1B* gene could be a risk factor for chronic periodontitis in the Brazilian population. We believe that the identification of genetic markers for susceptibility to periodontitis will allow an early identification of individuals with high risk, and could eventually help through individualized forms of therapy.

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